Role of CTCF in HOX genes regulation and breast cancer tumorigenesis

Muhammad Mustafa

Department of Medical Science

The Graduate School, Yonsei University

Role of CTCF in HOX genes regulation and breast cancer tumorigenesis

Directed by Professor Myoung Hee Kim

The Doctoral Dissertation
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Muhammad Mustafa

December 2015

This certifies that the Doctoral Dissertation of Muhammad Mustafa is approved

Thesis Supervisor: Myoung Hee Kim

Thesis Committee Member#1 : Hyoung Woo Park

Thesis Committee Member#2 : Kyoungsook Park

Thesis Committee Member#3 : Seung Il Kim

Thesis Committee Member#4 : Sang Sun Yoon

The Graduate School
Yonsei University

December 2015

ACKNOWLEDGMENTS

In the name of Lord, most Gracious, most Compassionate

Foremost, I would like to express my sincere gratitude to my advisor Professor Myoung Hee Kim for the academic and emotional support throughout my period of stay with her as a PhD student. I could not have imagined having a better advisor and mentor for my PhD studies. Thanks to her I had the opportunity to transform myself from a worker to a researcher with her valuable guidance, scholarly inputs and consistent encouragement. She spent many hours to install the missing tools of Molecular Biology and Epigenetics in me during the early phase of my PhD studies and then let me free to carry out my independent research projects. I believe that it might not be possible for me to discover my present state of mind without the gifted kindness and patience of my great advisor.

I would also like to thank my thesis committee member, Hyoung Woo Park, Kyoungsook Park, Seung Il Kim and Sang Sun Yoon for their valuable time and comments. I am feeling lucky to find the company of a teacher like Dr. Ji-Yeon Lee in the lab. Her expert opinions, comments and suggestions kept my research on track and saved so many of my working hours. I am also thankful to her for the translation of my thesis abstract in Korean language.

I strongly believe that seed of my success today was sown years ago with sweat and blood of a simple, honestly working man, my father, Muhammad Ismail. He worked day and night, between 0 to 50°C alone

and never thought of easing himself by sending me to work instead of school. I will stay in debt of all the efforts of my parents in raising me with the purest form of bread and butter. I am feeling great in writing thanks to my brother Ghulam Mohioudin and sisters for their support and specially my eldest sister Nazia who put her believe on my side during the most difficult times of my life.

It will be a different game all together, if I don't have Nousheen Mustafa as my wife. She is the wind beneath my wings during my journey of PhD studies. She provided excellent and comfortable environment for the family during the harshest of the times and equipped me with new energy and strength every day to compete with cutting edge research. My daughter Manal Mustafa worked as my stress reliever with her love and ever growing brilliance in science and education. I felt strong with the kindness of my boy Muhammad Ahmad Mustafa and lucky with the arrival of my daughter Fatima Mustafa, together with my nieces Eman and Dua, they made me believe that heavenly solutions are on their way for my social and research problems.

A very loving and proud part of my life belongs to my students who always believed that I was more than what I was doing as a teacher in their times. In particular I would like to mention Yasir Azeem, as a student, he was not meant to support his teacher but he worked as many characters as he could to ease my life, stood besides me and supported my journey during the thick and thin of my life.

I would like to thank all my friends for the great support and encouragement they provided, I was blessed specially with Muhammad

Arshad Awan, Farrukh Rasheed, Zahoor Khan and Usman Liaqat in Korea and with Abdul Rauf, Muhammad Shahid Nadeem, and Adil Khaleque, Muhammad Ahmad, Imran Safdar and many more in Pakistan.

My special thanks for Madam Debora, Martha and Joy Park for their great contribution in taking care of my family. They not only helped in day to day social matters but also provided the family relations which I missed in Korea. Together they enabled me to focus on my work without having much worries about my family and kids education.

Finally I would like to thank the generous funds provided by Higher Education Commission of Pakistan, without which I would never be able to reach Yonsei University for my higher studies.

Muhammad Mustafa

December 2015

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Role of CTCF in HOX genes regulation and breast cancer tumorigenesis

Muhammad Mustafa

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Myoung Hee Kim)

CTCF (or CCCTC-binding factor), a ubiquitous 11-zinc finger multifunctional protein has distinct molecular functions such as transcriptional activation, transcriptional repression, or enhancer blocking activity, in a locus-specific manner. Identification of somatic mutations in CTCF in different cancers and its involvement in cellular growth, differentiation and apoptosis point towards its role in cancer progression. HOX genes not only play important roles in defining body patterning during embryonic development, but also control numerous cellular events in adult cells. Deregulated *HOX* gene expression in different cancers including breast cancer is

now increasingly being reported.

In this work we wanted to investigate that how CTCF contribute in the breast cancer cell survival directly and through modulation of HOX genes. We performed CTCF gain and loss of function studies to see its effects on HOX genes expression and cancer cell survival and identified several directly regulated HOX genes including *HOXA4*, *HOXC8* and *HOXA10*. Among HOX, HOXA10 is an emerging tumor suppressor for its role in activation of p53 and in countering tumorigenesis in breast cancer. *HOXA10* silencing is associated with different cancers but the underlying mechanism is still elusive. Our data defines the putative promoter region of *HOXA10* 5.3-6.1 kb upstream of its start codon and its negative regulation by CTCF. Analysis of histone modification reveals that the presence of CTCF is associated with decreased active histone marks H3K4me3 and increased repressive histone marks H3K27me3 on *HOXA10* locus. Together with in silico analysis and specially designed in-vitro mutation assay we identified an important promoter element, flanked with CTCF core motif in *HOXA10* promoter region. Based on the evidence in our study, we propose that CTCF binding site co-exist within core promoter region of *HOXA10* and CTCF presence not only maintains the inactive state of local chromatin but also interfere with the transcription machinery and induce transcriptional silencing of *HOXA10*. Epigenetic silencing of *HOXA10* by CTCF in breast cancer cells may contribute towards tumorigenesis by avoiding cell cycle arrest, decreasing apoptosis and promoting metastasis.

We observed depletion of CTCF leads to decreased cell growth and proliferation in breast cancer cell line MCF-7 than normal breast epithelial cell line MCF10A. We found that it happens due to the direct and indirect activation of p53 signaling cascade in CTCF knock down breast cancer cells. CTCF binding near the promoter region of TP53 is marked with increased H3K27me3 and decreased H3K4me3. p53 activation by CTCF knock down resulted in cell cycle arrest at G1/S check point and enhanced apoptosis due to the activation of *p21* and *Bax* respectively. Nuclear localization of p53 is related with CTCF depletion in MCF-7 cells and it is consistent with our observation that p53 cascade is activated in the absence of CTCF. CTCF also induce transcriptional silencing of HOXA10 which is known to activate p53.We observed that HOXA10 co-localize with p53 in breast cancer cells and depletion of nuclear HOXA10 results in decreased nuclear p53. Several key components of autophagy signaling pathway e.g., ATG13, SQSTM1 (P62) and LC3-II as well as lysosome number are positively related to CTCF levels in MCF-7 cells.

Taken together we demonstrated that CTCF induce transcriptional silencing of p53 directly as well as through HOXA10 to contribute in the breast cancer survival and tumorigenesis.

Key words : breast cancer, CTCF, HOXA10, p53

Role of CTCF in HOX genes regulation and breast cancer tumorigenesis

Muhammad Mustafa

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Myoung Hee Kim)

I.INTRODUCTION

CCCTC binding factor (CTCF) is a highly conserved and ubiquitous transcription factor known to be involved in various unrelated cellular functions.[1, 2] The 82 kDa protein has three distinct domains: N-terminal, C-terminal and a central DNA binding 11-zinc finger domain.[3] CTCF uses different combinations of its 11-Zn-fingers in DNA binding domain to form different complexes for its multifunctional role in regulation of gene expression.[2] Its main functions are transcriptional activation, repression, and enhancer blocking depending on the genetic locus involved.[4-6] Many of the functions of CTCF involved long-range genome processes such as chromatin

looping, chromatin insulation and nuclear organization.[7-9] These are regulated by interactions with other protein partners and post translational modifications.[10] Originally discovered as a transcriptional repressor of chicken c-myc gene,[11] CTCF is also known to inhibit hTERT and *Bax* gene transcription by binding near to their promoter regions.[12, 13]

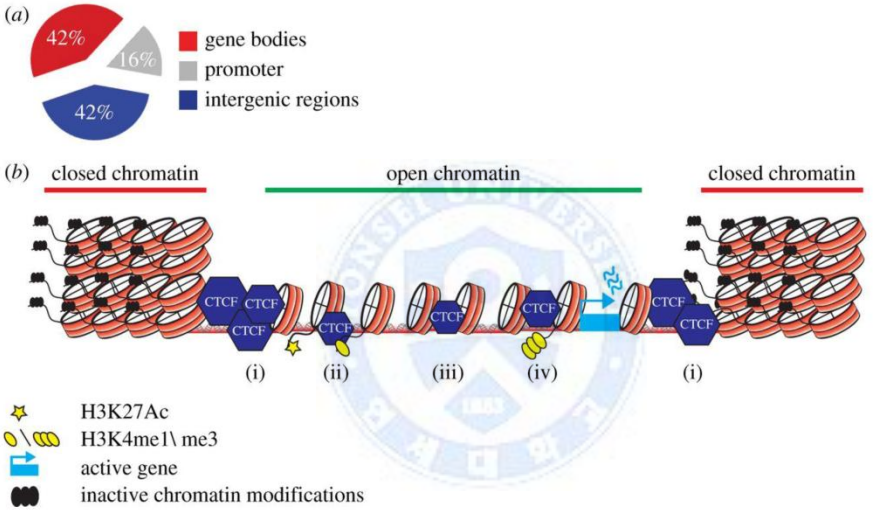


Figure 1. CTCF function in chromatin biology.[14] (*a*) Functional categories of CTCF binding sites across the genome (*b*) (i) CTCF binding sites are found at boundaries that separate active and inactive domains. CTCF binding to (ii) enhancer-like sequences and (iv) gene promoters can facilitate looping between these sequences. (iii) CTCF binding in between enhancers and gene promoters can block the interaction between an enhancer and its target promoter.

CTCF mutation is found in many cancers including breast cancer.[15] It regulates many cancer related genes involved in cellular growth, proliferation, differentiation and apoptosis.[16-20] Higher levels of CTCF was found in breast tumors and in breast cancer cell lines as compared to the normal cells and is associated with resistance towards apoptosis.[21] CTCF counteract the influence of positive regulators on *Bax* promoter and its depletion activates *Bax* transcription and promotes apoptosis in breast cancer cell lines.[13] The growing evidences over the years support the idea that higher levels of CTCF in breast cancer cells contribute in cancer cell survival by transcriptional repression of one or many important tumor suppressor genes like *Bax*. Therefore it is important to investigate the role of CTCF in regulation of cancer related genes to unveil the effect on breast cancer tumorigenesis.

HOX constitute a family of transcription factors that play key roles in embryonic development. They are characterized by a highly conserved homeodomain region consisting of a 60-amino acid motif which enables HOX proteins to bind to specific regions of DNA in order to transcriptionally activate or repress target genes.[22] In mammals, the 39 *HOX* genes organized into 4 paralogous groups (A, B, C and D) located on 4 different chromosomes.[23] During development, HOX gene expression is regulated spatially and temporally, followed by a strict code to influence patterning of the anterior to posterior axis. In adults, the expression pattern of HOX genes were conserved and maintained in a tissue specific manner[24] and gave unique morphological identities to the cells.[25, 26]

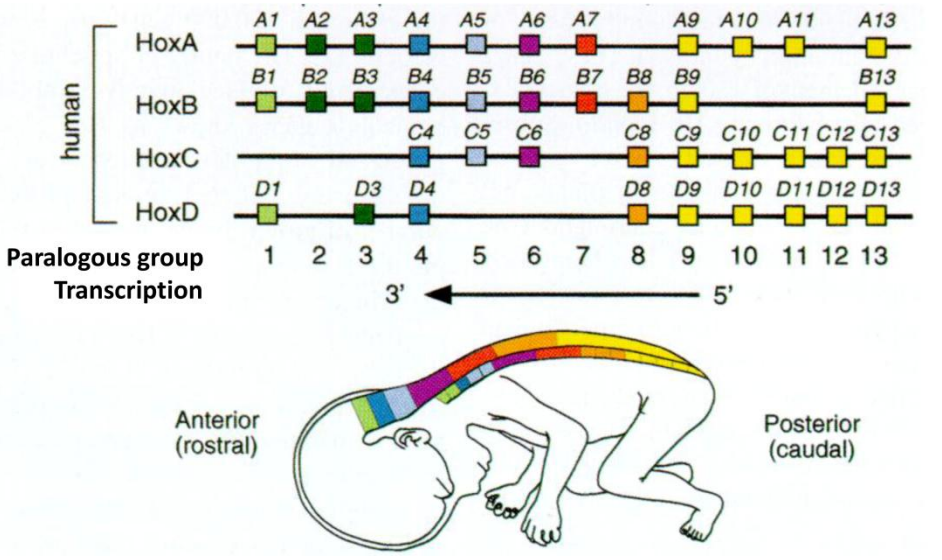


Figure 2. Genomic organization of human HOX genes and their collinear expression during development.[27]

HOX proteins regulate several key cellular functions such as cell adhesion, migration and cell cycle to maintain cellular homeostasis,[22] however, many HOX genes are found to be deregulated in different cancers and some of them have already established their roles in breast cancer tumorigenesis and progression.[28-35]

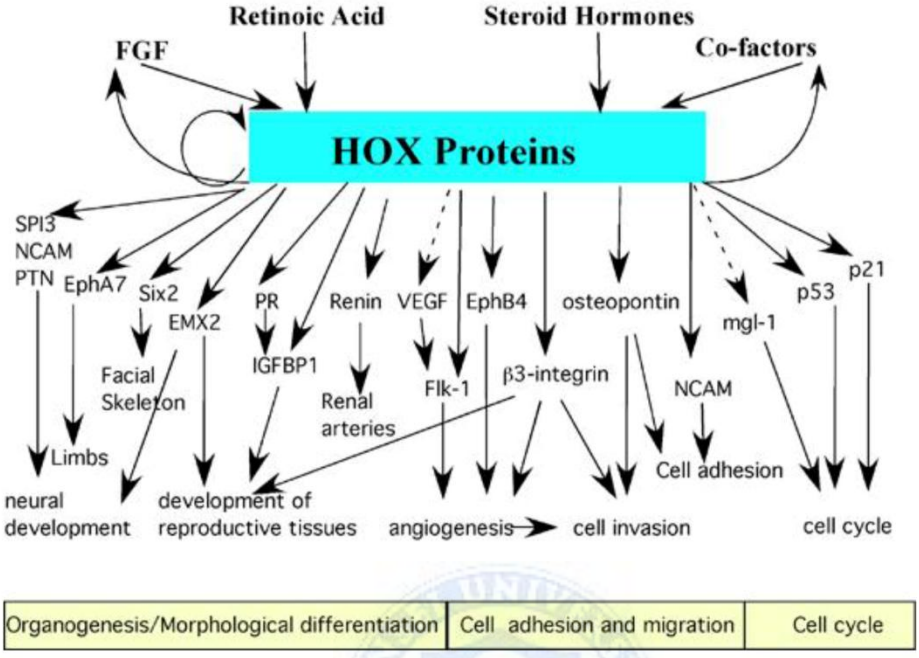


Figure 3. Downstream targets of HOX proteins. HOX downstream targets are involved in numerous cellular processes including organogenesis, cellular differentiation, cell adhesion and migration, cell cycle and apoptosis. Several downstream targets play multiple roles in several pathways, with many acting as transcription factors and regulating their own subset of genes.[36]

Many HOX genes including an emerging tumor suppressor HOXA10 is silenced in patients with hereditary breast cancer.[37] During development, HOXA10 controls uterine organogenesis and also functions for endometrial differentiation in adult.[38] In endometrial carcinoma, downregulation of *HOXA10* by promoter methylation is associated with increased tumor grade.[39] In differentiating myelomonocytic cells

(U937), HOXA10 binds to the promoter region of p21 and activate its transcription which results in cell cycle arrest and differentiation.[40] HOXA10 is also known to regulate p53 in breast cancer cells[34] and its down-regulation increases cell migration and metastasis.[26] These studies strengthen the role of HOXA10 as a tumor suppressor in breast cancer cells and propose its activation as a potential treatment but the molecular mechanism of how HOXA10 is regulated in breast cancer cells is still elusive.

Human HOX gene clusters contain several CTCF binding sites which establish discrete functional chromatin domains. Deletion of CTCF binding site(s) within HOX cluster results in expansion of active chromatin into the repressive domains.[41] CTCF functions as a controller of HOXA cluster silencing and mediate PRC2-repressive higher-order chromatin structure.[42] The ability of CTCF to modulate HOX expression pattern will subsequently affects the downstream signaling cascades and contributes in cancer development.

The higher level of CTCF in breast cancer cell lines and the availability of huge number of CTCF binding sites in human genome[43] indicate that it might involve in the silencing of several other tumor suppressor gene on a global scale. We investigate the role of CTCF in breast cancer cell progression directly or by modulating HOX gene expression. By using ER/PR positive (MCF-7, BT-474 and T-47D) breast cancer cell lines and normal breast epithelial cell line (MCF10A) we demonstrate that CTCF depletion affects breast cancer proliferation by a combinatorial effect of cell cycle

arrest, increased apoptosis and blocking autophagy. CTCF also suppress the transcription of several important HOX tumor suppressors like *HOXA4*, *HOXA10* and *HOXC8* and contribute in breast cancer tumorigenesis. Targeted deletions of CTCF binding sites to reduce CTCF enrichment near the promoter region of tumor suppressor genes like *HOXA10* will activate cellular defense mechanism and can induce apoptosis. The normal breast cells which are not much dependent on CTCF might be spared and can survive with this type of targeted therapy

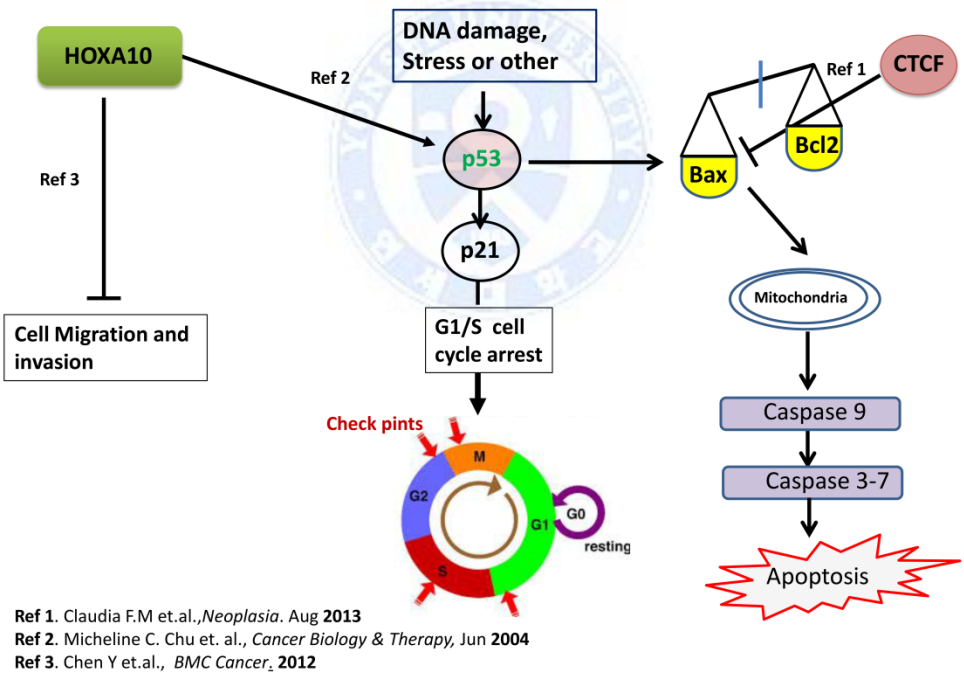


Figure 4. p53 signaling cascade. p53 signaling pathway can be activated on DNA

damage and other types of stress as well as by HOXA10. Activation of p53 results in cell cycle arrest and apoptosis by upregulation of p21 (CDKN1A) and Bax respectively.

II. Materials and Methods

1. Cell Lines and Cell Culture

MCF-7, BT-474 and T-47D cells were kindly provided by Drs. Young Nym Kim and Kyung Tae Kim (National Cancer Center Korea). All the breast cancer cell lines were maintained at 37^{o}C in an atmosphere of 5% CO_2 using following conditions. MCF-7, BT-474 were cultured in Dulbecco's modified Eagle's medium (DMEM; WelGENE Inc, Daegu Korea) and T-47D cells were cultured in RPMI 1640 (WelGENE Inc). The media were supplemented with 10% fetal bovine serum (FBS; WelGENE Inc) and 1X antibiotic antimycotic solution (WelGENE Inc).

2. RNA Isolation and RT-PCR

Total RNA from the cultured cells was isolated using Trizol reagent (Invitrogen Carlsbad, CA USA) according to the manufacturer's instruction. 500 ng of total RNA was reverse transcribed (RT) using ImProm-II^{TM} Reverse Transcriptase (Promega, Madison, WI, USA). PCR was performed in replicates using G Taq polymerase (Cosmogenetech, Seoul Korea). Following PCR conditions were used for amplification: initial denaturation for 3 min at 95^{o}C followed by 25-35 cycles (25 cycles for β-Actin, 32 cycles for CTCF and 35 cycles for HOXA genes) of 95^{o}C for 30 sec 58-60^{o}C (58^{o}C for β-Actin, 60^{o}C for CTCF and HOXA genes) for 30 sec, 72^{o}C for 30 sec. Final elongation was done at 72^{o}C for 5 min. PCR primers for HOX genes

were previously reported.[44] Primers for other genes were listed in Table 3. For quantification, ImageJ software was used. Minimum three independent biological replicates were analyzed and Student's t-test was used to calculate *p*-value wherever required.

3. Overexpression of CTCF

Full length CTCF cDNA was cloned into pCDNA3 vector to obtain pCTCF recombinant plasmid. Human breast cancer cell lines were transfected with either pCDNA3 control or pCTCF plasmid DNA using Lipofectamin 2000™ (Invitrogen) or Attractene (QIAGEN) according to the manufacturer's protocol. Transient overexpression of CTCF was confirmed after 72 hr at mRNA level by RT-PCR and at protein level by western blot analysis using CTCF antibody (Cell signaling, 3418).

4. Knockdown of CTCF

shRNA oligos (Table 1) targeting two different positions on CTCF mRNA (shCTCF1, gcggaaagtgaacccatgata and shCTCF2, cctcctgaggaatcaccttaa) were designed and synthesized by Cosmo GENETECH. Oligos were annealed to generate shRNA inserts with *AgeI* and *EcoRI* sites, and cloned into pLKO.1 puro to generate pLKO.1-shCTCF1 and pLKO.1-shCTCF2. Packaging plasmid (pCMV-dR8.91) and envelop plasmid (pVSV-G) were co-transfected with control (pLKO.1) or Non-specific shRNA containing vector (pLKO1-NS) or shCTCF containing vector

(pLKO1-shCTCF1 or pLKO1-shCTCF2) using Lipofectamin 2000™ (Invitrogen) in HEK-293T cells. Lentiviruses from the media were harvested twice after 48 hr and 72 hr, pooled, filtered (Minisart ® 0.45 µM CE) and saved at -20 °C. For CTCF knock down, breast cancer cells were grown in media containing lentivirus particles (1-10 dilution) for 72 hr in the presence of 8 µg/mL polybrene (Sigma). Selection was done using 1.5µg/mL puromycine for 48 hr and CTCF knockdown was confirmed by RT-PCR.

5. Dual Luciferase Assay

Different genomic DNA fragments (Table 2) from HOXA10 locus were amplified using pfu polymerase (Solgent) and cloned into pGL3-basic vector (Promega) using *KpnI* and *HindIII* sites. Restriction enzyme sites were incorporated via forward and reverse primers (Table 2). HEK-293T and MCF-7 cells were co-transfected with Renilla luciferase vector (pRL Renilla luciferase control) and either of control pGL3-basic vector or individual luciferase genomic construct using Attractene (QIAGEN) according to the manufacturer's protocol. Promoter activity of individual construct was measured using Dual luciferase® assay system (Promega) after 72 hr of transfection and normalized using Renilla luciferase signal. To analyze the effect of CTCF on the promoter activity, MCF-7 cells were co-transfected with Renilla luciferase vector and pGL3-A10-2 vector with either pCDNA3 or pCTCF vector and promoter activities were measured as previously described. Minimum 4 independent

biological replicates were examined to obtain reliable results.

6. In-vitro mutation assay (IMA)

Wild type pGL3-A10-2 plasmid was mutated using PCR based introduction of mutation(s) to generate pGL3-A10-2-m1, pGL3-A10-2-m2 and pGL3-A10-2-m3 plasmids. Primers with mutated sequences F2 and R2 were used to amplify DNA fragments with their corresponding F1 and R1 primers according to the scheme in Figure 14 (Figure 14A). Resulting DNA sub fragment were purified and 1 µl of equimolar mixture of sub-fragments were used as a template for assembly PCR with F1 and R1 primers to generate mutated A10-2 fragment (Figure 14B). A10-2-mutated fragments are later cloned back into pGL3-basic vector for Dual luciferase assay (Figure 14C).

7. Chromatin Immunoprecipitation (ChIP) Analysis

Chromatin for ChIP experiment was prepared from MCF-7, BT-474 and T-47D cells by fixing the cells in 37 % formaldehyde for 15 min, followed by quenching with 2.5 M Glycine for 10 min. Sonication of cross linked genomic DNA was done using Sonics Vibra Cell^{TM} to get 500~1000bp DNA fragments (Figure 13A) with condition 1 (Time: 7 min, Pulse: 10 sec Interval 10 sec). In case of modified ChIP experiment (Figure 13B) DNA fragment up to 250bp were generated using Condition 2 and 3 (Time: 9 min and 11 min, respectively, for condition 2 and 3 with

same pulse and interval as condition 1). ChIP was performed using antibodies against CTCF, H3K4me3 (abcam, 1012), H3K27me3 (abcam, 6002) and IgG (Santa Cruz Biotechnology, sc 2027). Immune precipitated DNA was extracted using Cosmogenetech Labopass^{TM} PCR purification kit. Two μl of eluted DNA was used as a template to perform PCR, DNA was resolved on 1.5% agarose and quantified using ImageJ software. The primers corresponding to CBS1 and CBS2 sites (Figure 12) are given in Table 3. Minimum three or more independent biological replicates were used for quantitation.

8. Immunocytochemistry

MCF-7, BT474 and T-47D were fixed using 4% paraformaldehyde (PFA) for 15 min, rinse with cold Phosphate buffer saline PBS twice and permeabilized with 0.25% Triton-X100 in PBS for 10 min. The cells were washed with PBS 3 times for 5 min. Blocking was done using 1% BSA in Phosphate buffer saline with 0.1% Tween PBST for 30 min. In case of HOXA10 and CTCF, cells were washed with PBS 3 times for 5 min. Cells were incubated overnight at 4^{o}C in a humidified chamber with primary antibody (HOXA10 N-20: Sc-17158 Goat polyclonal and/or CTCF Rabbit monoclonal antibody (Cell signaling, 3418) diluted in blocking buffer at 1:50. After washing with PBS three times for 5 min, cells were incubated with secondary antibody (Donkey Anti-Goat IgG Alexa Flour® 488, ab150129 and/or Goat Anti-Rabbit IgG Alexa Flour 594 (ab150084)) in blocking buffer for 1 hr in dark. In case of

p53 and CTCF, primary antibodies p53 (DO-1) sc-126 and CTCF Rabbit monoclonal antibody (Cell signaling, 3418) were detected by secondary antibodies Goat Anti-Mouse IgG Alexa Flour 488 (ab150117) and Goat Anti-Rabbit IgG Alexa Flour 594 (ab150084). In case of p53 and HOXA10, primary antibodies p53 (DO-1) sc-126 and HOXA10 N-20: Sc-17158 Goat polyclonal were detected by secondary antibodies Goat Anti-Mouse IgG Alexa Flour 488 (ab150117) and Rabbit Anti-Goat IgG Alexa Flour 568 (ab175707). Counter stain with 0.5 µg/ml DAPI (invitrogen™) for 10 min and analyzed using Zeiss LSM700 Confocal microscope.

9. MTT Assay

5000 cells per well in 96-well plate were 24 hr before treatment with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). 20 µl of 5mg/mL MTT (in PBS) was added to each well, incubated for 3.5 hr at 37°C. Removed media and added 150 µl of MTT solvent (4mM HCL, 0.1% Nondet P-40 (NP-40) in Iso-propanol). Kept on shaker for 15 min and read at absorbance at 570 nm in Elisa Plate Reader.

10. Cell Cycle Analysis

3.0 x 10^5 cells were harvested and fixed using 2ml of 70% Ethanol, kept at 4°C for 2.30 hr. Ethanol was removed and cells were washed twice with PBS and re-suspended in 475 µl of PBS. Cells were passed through syringe to avoid aggregation,

5 ul RNAse A (10mg/ml) and 25 µl of PI (1mg/ml) were added. Incubated the cells at 37°C in dark for 45 min and analyzed using Fluorescence Activated Cell Sorter (FACS).

11. Apoptosis Analysis

EzWay Annexin V-FITC Apoptosis Detection Kit (KOMABIOTECH) was uded to detect apoptosis. 5 x ^{105} cells were transferred in microtube, centrifuged, washed with 0.5mL cold PBS twice and with 0.5mL cold Binding buffer. Added 1.25 µl (200 µg/ml) of Annexin V-FITC and incubated at room temperature for 15 min in dark. Removed the supernatant and washed cells with 0.5mL cold Binding buffer. Added 10 µl of PI (30 µg/ml) to the cells suspension and analyzed by FACS.

12. Autophagy detection by Lysotracker

Lysotracker DND-26 (Cell signaling 8783) were diluted 1:10,000 directly into cell culture media to make 100 nM final concentration. Live cells were analyzed using fluorescent microscopy immediately after treatment without fixation.

13. PCR array

RNA samples from CTCF knock down and control MCF-7 cells were used for Human Breast Cancer RT² Profiler™ PCR Array system by SABiosciences.

14. Statistical analysis

For all the experiments, minimum three independent biological replicates were analyzed for quantitation. Data are presented as mean ± standard error. Student's t-test was used.

III. RESULTS

1. CTCF regulate expression of HOX genes in MCF-7

CTCF ChIP-Seq in MCF-7 cells[45] adopted from Encode genome browser showed that several CTCF binding sites were present in HOX cluster (Figure 5). This indicates a possible role of CTCF in regulation and maintenance of HOX cluster expression pattern in MCF-7.

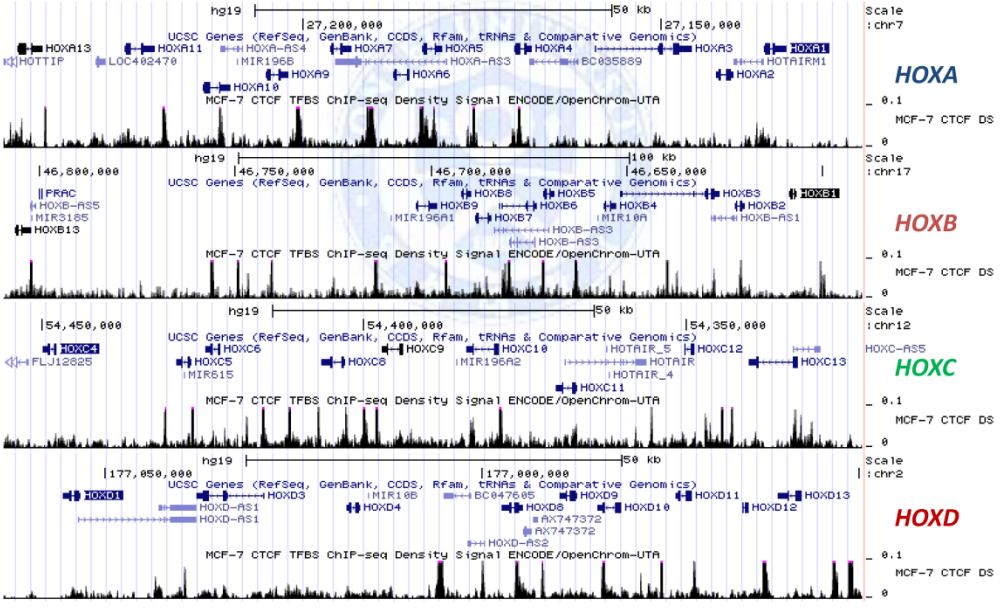


Figure 5, CTCF binding sites within HOX genes cluster[45] in MCF-7 cells adopted from Encode genome browser.

In order to find a strong candidate gene regulated by CTCF among HOX, we first performed gain and loss of function study and examined the effect of CTCF on the expression of HOX cluster genes (Figure 6-8). The overexpression of CTCF in MCF-7 was validated at mRNA level by RT-PCR (6A) and at protein level by western blotting (Figure 6B). Expression of HOX genes were analyzed using semi-quantitative RT-PCR (Figure 6C-8A). *HOXA5* was upregulated while *HOXA4*, *HOXA10* and *HOXC8* were downregulated in CTCF over-expressing MCF-7 cells (Figure 6C), Others were unchanged. When CTCF was knocked down using lentivirus (Figure 7A,B) and expression pattern of HOX genes were analyzed, it was found that HOXA4, *HOXA10* and *HOXC8* were upregulated (Figure 7C). Semi-quantitative PCR analysis confirmed that expression of *HOXA4*, *HOXA10* and *HOXC8* were inversely related to CTCF levels in MCF-7 cells (Figure 8B).

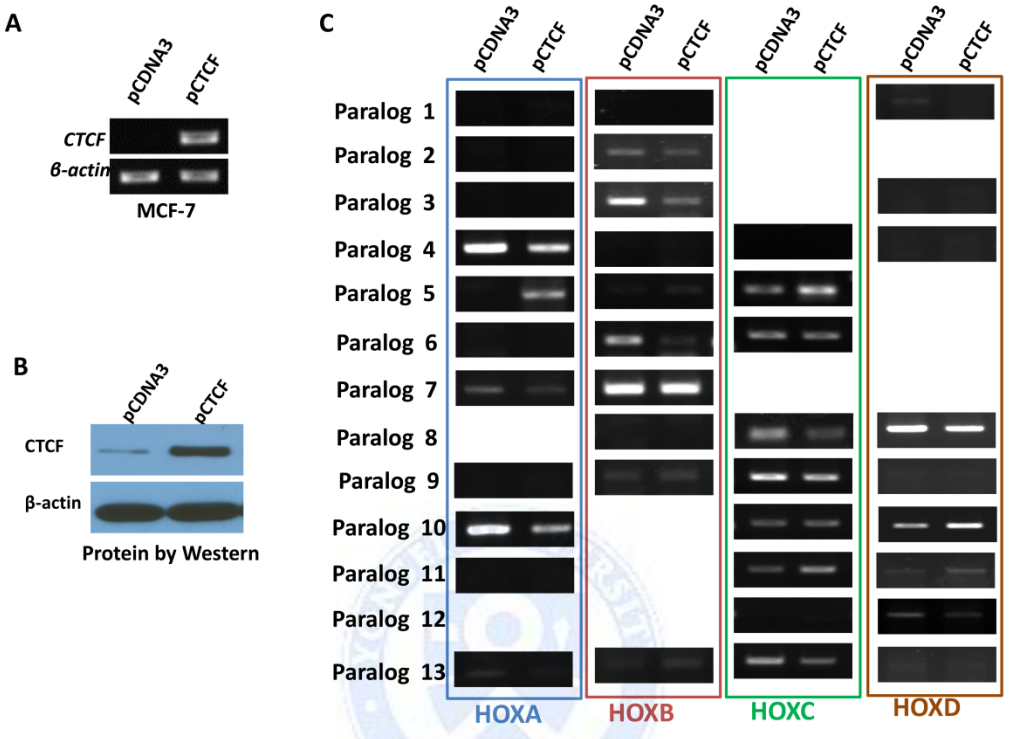


Figure 6. Effect of CTCF overexpression on the expression pattern of HOX genes in MCF-7 cells. (A) mRNA levels of CTCF in control (pCDNA3) and CTCF over expressing (pCTCF) MCF-7 cells (B) Western blot analysis of control and CTCF overexpressing MCF-7 cells. (C) A representative data showing up or down regulation of HOX genes in CTCF overexpressing cells.

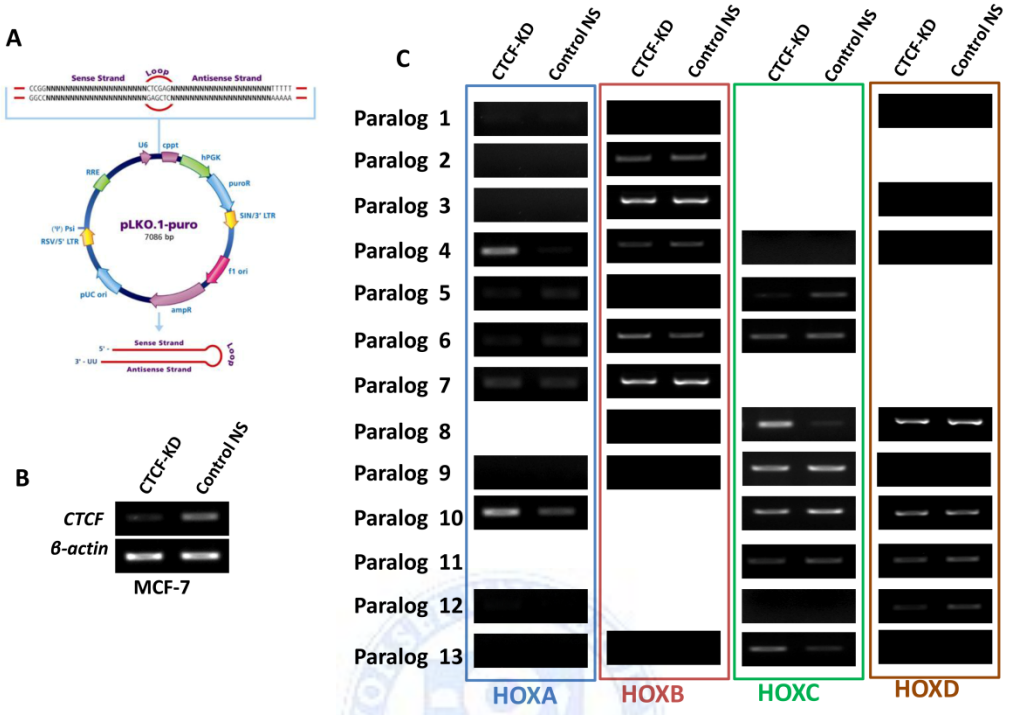


Figure 7. Effect of CTCF knock down on the expression pattern of HOX genes in MCF-7 cells. (A) Representation of pLKO.1-puro vector used to generate shRNA against CTCF mRNA (B)) mRNA levels of CTCF in control non-specific (NS) and CTCF knock down (KD) MCF-7 cells (C) A representative data showing up or down regulation of HOX genes in CTCF knock down MCF-7 cells.

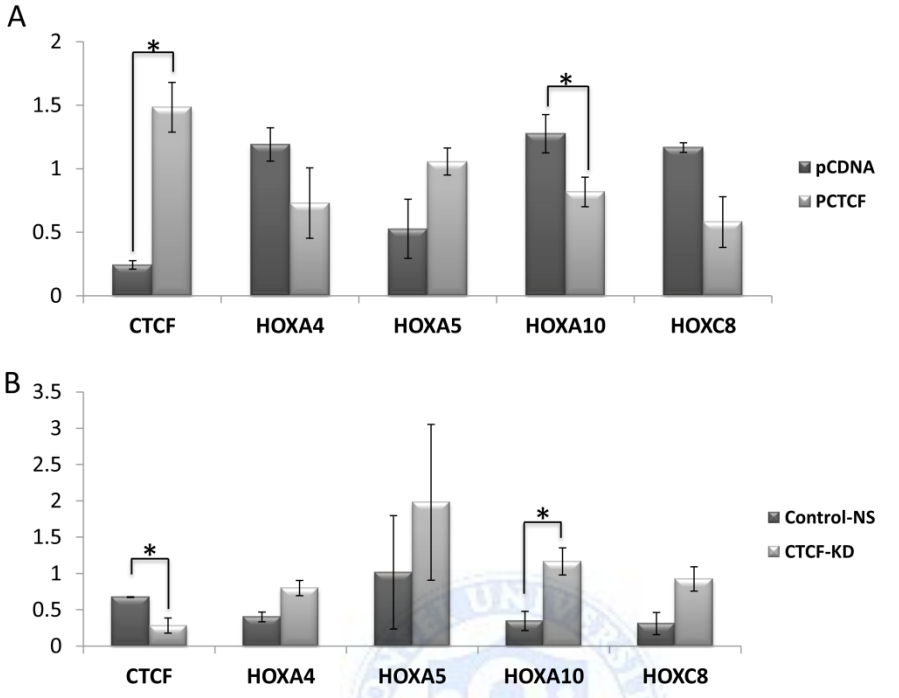


Figure 8. Semi-quantitative RT-PCR data of candidate HOX genes in CTCF over expressing and knock down MCF-7 cells. (A) Result of semi-quantitative RT-PCR showing the change in expression pattern of selected genes in CTCF overexpressing cells. Error bars are standard error, n = 4. *p<0.01. (B) RT-PCR analysis of candidate gene in CTCF knockdown cells. Error bars are standard error, n = 4. *p<0.05

Table 1. Synthetic shRNA oligos for CTCF knock down

Oligo	Sequence
shCTCF1-F	5'CCGGGCGGAAAGTGAACCCATGATACTCGAGTATCATGGGTTCACTTTCCGCTTTTTG
shCTCF1-R	5'AATTCAAAAAGCGGAAAGTGAACCCATGATACTCGAGTATCATGGGTTCACTTTCCGC
shCTCF2-F	5'CCGGCCTCCTGAGGAATCACCTTAACTCGAGTTAAGGTGATTCCTCAGGAGGTTTTTG
shCTCF2-R	5'AATTCAAAAACCTCCTGAGGAATCACCTTAACTCGAGTTAAGGTGATTCCTCAGGAGG

Among three candidate HOX genes, HOXA10, in particular, has been proposed as a tumor suppressor gene but not well studied for its regulation mechanism, we therefore selected *HOXA10* as a candidate gene to explore further for its regulation by CTCF. ICC data proved that HOXA10 nuclear and cytoplasmic expression was also increased after CTCF knockdown in MCF-7 cells (Figure 9).

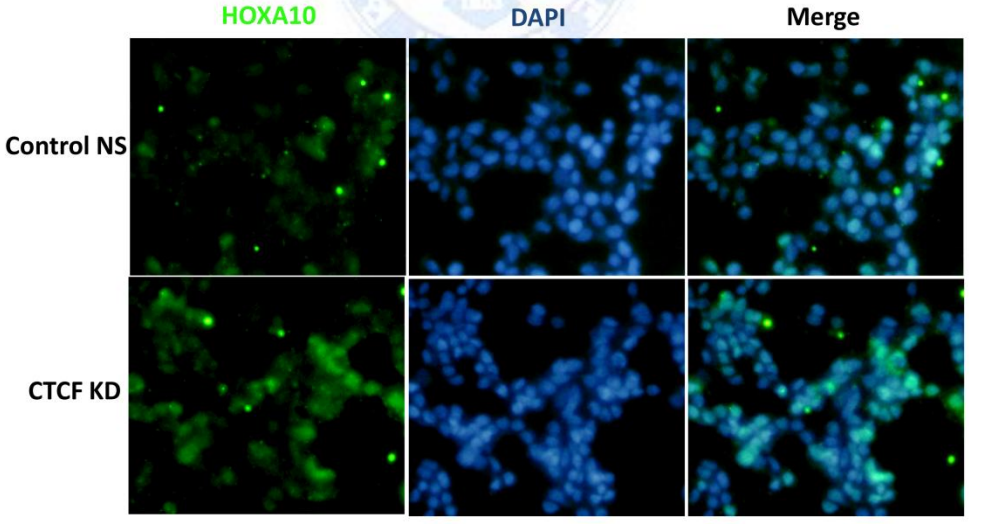


Figure 9. Immunocytochemistry (ICC) data showing HOXA10 localization in control

non-specific (NS) and CTCF knockdown (KD) MCF-7 cells.

2. Identification of promoter region of HOXA10 and effect of CTCF on promoter activity

To investigate whether CTCF regulates *HOXA10* expression directly by binding to its promoter region, we first performed a promoter assay using different gnomic DNA fragments from region 1 (A10-2; -268 to +507) and region 2 (A10-3; +4589 to +5861) of *HOXA10* locus (Figure 10A and Table 2). To our surprise, no promoter activity was observed from region 2 of *HOXA10* locus, which appears to be a typical regulatory region in most of the protein coding genes. Instead we found a significant promoter activity with A10-2 DNA fragment from region 1 (Figure 10B). Serial deletion of A10-2 fragment proved that entire promoter activity can be detected only with the full length of A10-2 fragment as deletion from both ends caused significant loss of promoter activity (Figure 10B). Similar results were obtained when the constructs were transformed in HEK-293T cells (Figure 10C).

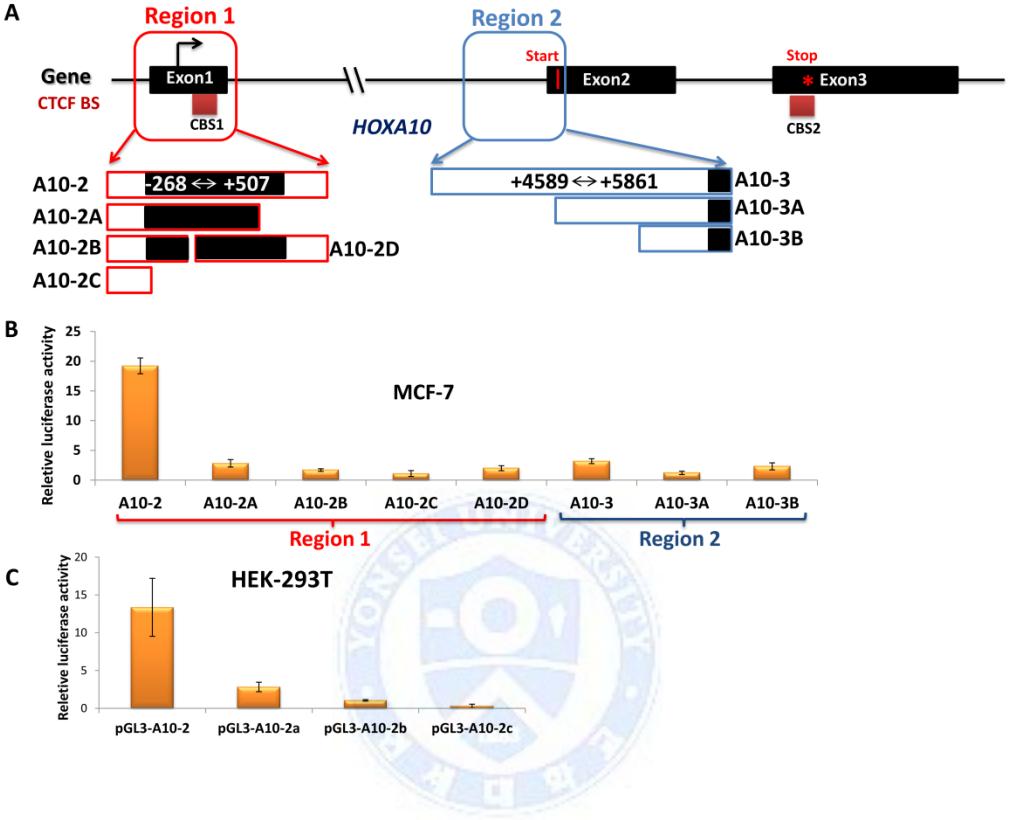


Figure 10. Identification of promoter region of *HOXA10*. (A) Representation of *HOXA10* locus (Figures not drawn to scale) showing all three exons with CTCF binding sites on exon 1 and exon 3. Start and stop codons are marked with red bar and asterisk, respectively. Genomic positions of DNA fragments of *HOXA10* region 1 and region 2 (red and blue boxes, respectively) for dual luciferase promoter assay are represented with their deletion constructs. (B) Promoter activities of individual constructs by dual luciferase reporter assay. Error bars are standard error, n=4. (C)

Relative luciferase reporter activities of different genomic DNA constructs in pGL3-basic vector normalized with Renilla luciferase vector (pRL-TK) in HEK-293 cells. n=3 and error bars are SE

Table 2. Primers for the amplification of genomic constructs for luciferase assay. Genomic constructs with their product sizes and genomic positions as represented in Figure 10A, *KpnI* and *HindIII* sites are in bold

Fragment	Forward primer	Size (bp)	Genomic location
	Reverse primer		
A10-2	5'-AGA**GGTACC**AGGGTAGTGGGACAGATCT -3'	**775**	**chr7:27,219,252-27,220,026**
	5'-AGA**AAGCTT**TGTGGCCTCTTACCTTGAC-3'		
A10-2A	5'-AGA**GGTACC**AGGGTAGTGGGACAGATCT-3'	**575**	**chr7:27,219,452-27,220,026**
	5'-AGA**AAGCTT**AGAAGCGAACAAAGGCC-3'		
A10-2B	5'-AGA**GGTACC**AGGGTAGTGGGACAGATCT-3'	**375**	**chr7:27,219,652-27,220,026**
	5'-AGA**AAGCTT**ATCTCATTTTTACATCTAAGAA-3'		
A10-2C	5'-AGA**GGTACC**AGGGTAGTGGGACAGATCT-3'	**147**	**chr7:27,219,880-27,220,026**
	5'-AGA**AAGCTT**TAATCTTTAACCATCCCAAA-3'		
A10-2D	5'-AGA**GGTACC**TGAGATCTCAATAGCAGCG-3'	**406**	**chr7:27,219,252-27,219,657**
	5'-AGA**AAGCTT**TGTGGCCTCTTACCTTGAC-3'		
A10-3	5'-AGA**GGTACC**AGGGCCTGACCTCCACAT-3'	**1272**	**chr7:27,213,898-27,215,169**
	5'-AGA**AAGCTT**GGAGCAGATAGCCCTTTC-3'		
A10-3A	5'-AGA**GGTACC**GGGAACAACCACTGCATT-3'	**684**	**chr7:27,213,898-27,214,581**
	5'-AGA**AAGCTT**GGAGCAGATAGCCCTTTC-3'		
A10-3B	5'-AGA**GGTACC**GTATGGGGCTCACGTCCG-3'	**336**	**chr7:27,213,898-27,214,233**
	5'-AGA**AAGCTT**GGAGCAGATAGCCCTTTC-3'		

When CTCF is overexpressed in MCF-7 cells (Figure 11A), the promoter activity of A10-2 fragment was decreased (Figure 11B). The negative effect of CTCF on the promoter activity of A10-2 fragment was observed in a dose-dependent manner

(Figure 11C). Taken together, we concluded that the promoter activity for HOXA10 transcription lies 5.3 to 6.1 kb upstream of the start codon of *HOXA10* (chr7:27,219,252-27,220,026) and is negatively regulated by CTCF.

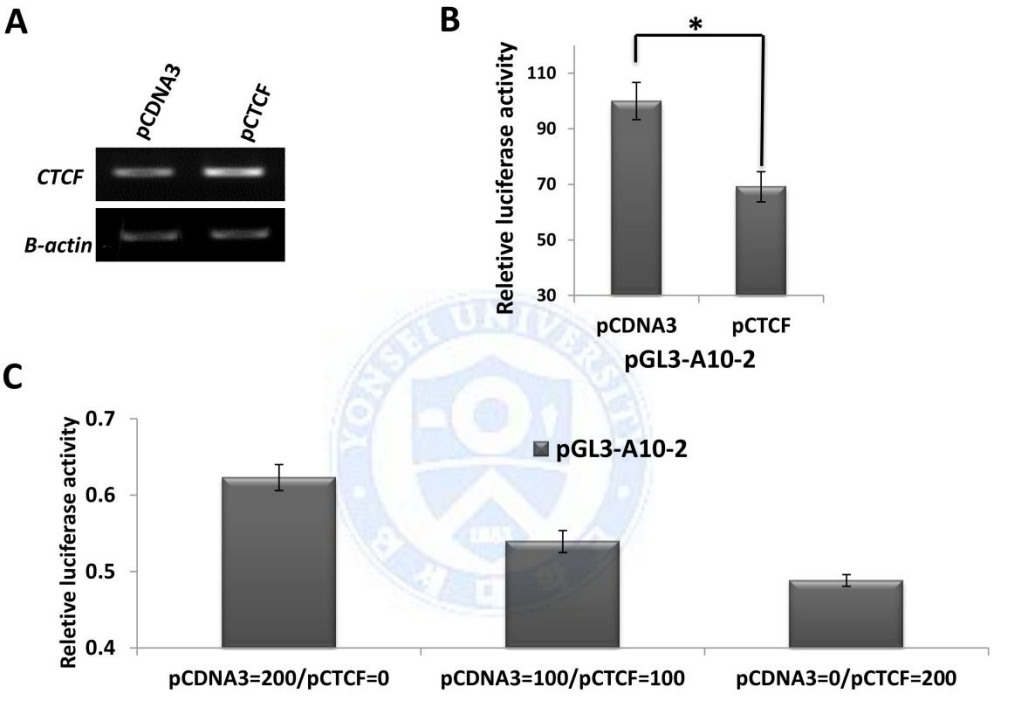


Figure 11. Effect of CTCF on *HOXA10* promoter activity. (A) mRNA expression level of *CTCF* in MCF-7 cells expressing empty vector or CTCF expressing vector with pGL3-A10-2. (B) Relative promoter activities of A10-2 fragment in control pCDNA3 set as 100 and CTCF expression vector. Error bars are standard error, n=4, *$p < 0.01$. (C) CTCF dose response on the promoter activity of A10-2 DNA fragment

with varying amounts (0-200 ng) of pCDNA3 control and pCTCF expression vectors.

3. CTCF binding to HOXA10 promoter region maintains inactive local chromatin state

According to the ChIP-Seq data[45] uploaded in ENCODE genome browser (Figure 5), *HOXA10* locus contains two CTCF binding sites (CBS) i.e., CBS1 and CBS2, found in exon 1 and exon 3, respectively. Each of the two CTCF binding sites was confirmed by ChIP-PCR with two sets of primers A and B spanning approximately 200 bp (Figure 12A, Table 3). Interestingly, we found that CTCF binds preferentially to CBS1-A site which contains the promoter activity (Figure 12B). Our finding of coexistence of putative regulatory region of *HOXA10* with the CTCF binding site CBS1 strongly suggests a possibility of direct role of CTCF in *HOXA10* promoter activity. To confirm whether the CTCF affect the promoter activity, we investigated the enrichment status of active and repressive histone markers (H3K4me3 and H3K27me3) at CBS1. CBS2, which is present within the exon 3, was also included to observe the effect of CTCF on the gene body. ChIP analysis for histone modification patterns revealed that the promoter region of *HOXA10* bound by CTCF is marked with higher H3K27me3 not only on the promoter region (CBS1-A) but also in the gene body (CBS2-A). Depletion of CTCF on *HOXA10* promoter region is associated with decreased H3K27me3 and slight increase of H3K4me3 (Figure 12C, D). These results suggest that CTCF binding to the promoter region of *HOXA10* confers

transcriptional silencing by maintaining inactive local chromatin state with higher enrichment of repressive histone marks H3K27me3.

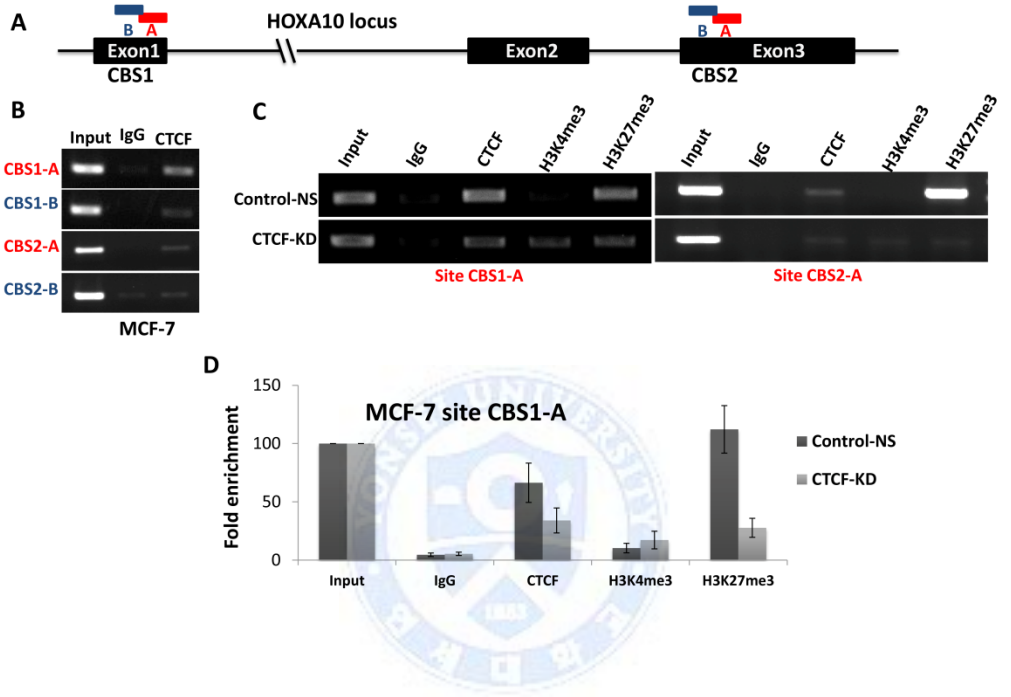


Figure 12. CTCF presence on *HOXA10* promoter is associated with repressive histone marks. (A) *HOXA10* gene locus with two CTCF binding sites (B) ChIP-PCR analysis of CTCF binding at site A and site B within CBS1 and CBS2 sites. (C) ChIP-PCR using control non-specific (Control-NS) and CTCF knockdown (CTCF-KD) MCF-7 cells to analyze histone modification on CTCF binding regions. (D) Quantitation of PCR results showing the effect of CTCF depletion on the enrichment of H3K4me3 and H3K27me3 at the site CBS1-A.

Table 3. RT-PCR primers for CTCF and Primers for ChIP analysis (Figure 12) and their corresponding genomic locations

Fragment	Forward primer Reverse primer	Genomic location
CTCF	5’ -TGCGAA AGCAGCATTCCTAT-3’ 5’ -TAGCGCTTG AAGTGCATG-3’	chr16:67,596,310-67,673,088
CBS1-A	5’ -AACTCCGGCCCAACCTAG-3’ 5’ -CATACCAATCACTTCTTGAGGGT-3’	chr7:27,219,360-27,219,565
CBS1-B	5’ -AATGCGCCGCTATAAACCC-3’ 5’ -GGCTTTGACATTGATCGGAAG-3’	chr7:27,219,501-27,219,712
CBS-2A	5’ -GAATCGAGAAAACCGGATCC-3’ 5’ -TCAGAACAAACCAGCCCT-3’	chr7:27,211,354-27,211,569
CBS-2B	5’ -AAGCACCAGACACTGGAG-3’ 5’ -TTAAAGTTGGCTGTGAGCTC-3’	chr7:27,211,528-27,211,718

4. Exploration of CTCF binding motif flanked with important promoter element of *HOXA10*

Our ChIP-PCR data confirmed the presence of CTCF binding within the region for promoter activity. Using CTCFBSDB 2.0, an online tool, we identified the core CTCF binding motif within putative promoter region A10-2 (Figure 15A). The identified motif has significant similarity with already established sequences of CTCF core motif (Figure 15B). The binding of CTCF on the exact motif was validated using modified ChIP experimental scheme with DNA fragments sonicated up to 250 bp approximately (Figure 13).

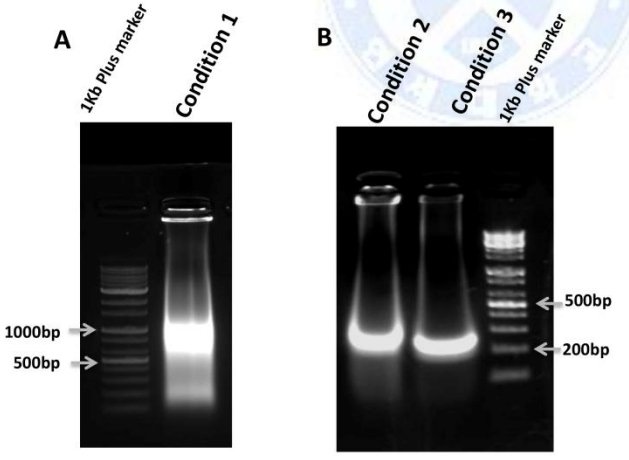


Figure 13. Sonicated DNA fragments for ChIP and modified ChIP experiment using MCF-7 cells. (A) Sonicated fragments ranged 500-1,000 bp with typical protocol of

ChIP method. (B) Extensive sonication reduced chromatin fragments to an average size of 250 bp (detailed protocols are in materials and methods).

PCR product was observed from the primers P1/P2 with predicted CTCF binding motif, but not with P3/P4, adjacent region without CTCF core motif (Figure 15C). We next asked whether this CTCF core motif is overlapped with important promoter regulatory region. To get the answer, we generated three mutated A10-2 fragments with mutations in CTCF core binding motif (A10-2-m1, m2, and m3; Figure 15D, Table 4) using the scheme (Figure 14) and measured the changes in promoter activity.

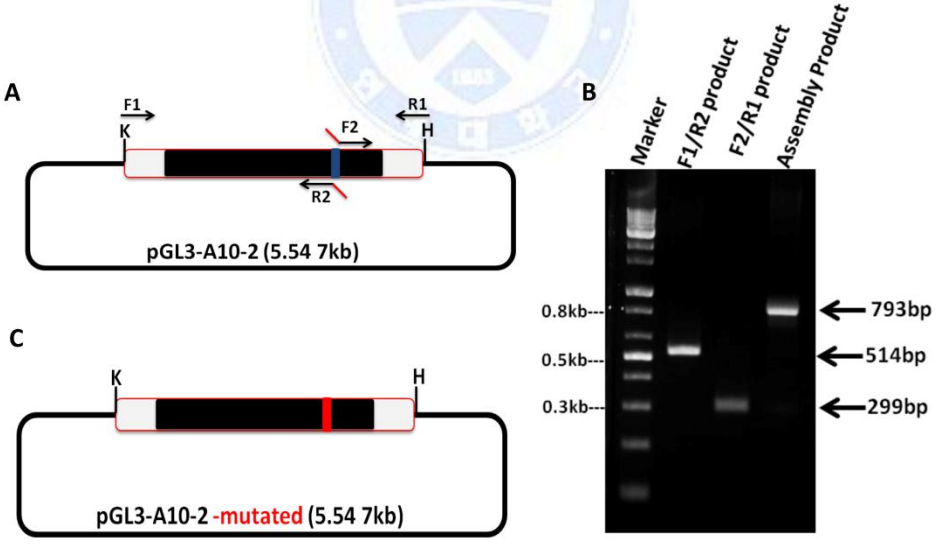


Figure 14. Scheme for the generation of pGL3-A10-2 mutated plasmids for IMA. (A) Primers with mutated sequences (red) flanking core CTCF binding motif (blue box) were used to generate mutated DNA fragments. K and H represent *Kpn*I and *Hin*dIII enzyme sites. (B) PCR products resolved on 1.5% agarose gel showing, marker (Lane 1), 514 bp F1/R2 (Lane 2) and 299 bp F2/R1 (Lane 3) products, along with purified assembly PCR product of mutated A10-2 (Lane 4). (C) Cloning of assembly PCR product using enzyme sites *Kpn*I and *Hin*dIII to produce pGL3-A10-2 mutated DNA plasmids for dual luciferase assay.

Dual luciferase reporter assay with wild type A10-2 fragment showed that promoter activity was not affected with fragment A10-2-m1 which contains only one nucleotide mutation. However, a fragment A10-2-m2 harboring 2 nucleotides changes, as well as a fragment A10-2-m3 with many changes within 20 bp CTCF core motif, showed significant reduction in promoter activity (Figure 15E). An online tool named "Neural network promoter prediction" predicted TATA box at -24 and the transcription start site in A10-2 region, as indicated by arrow in Figure 15A. Taken together, we concluded that CTCF core binding motif resides 218 bp downstream of transcription start site (TSS) flanked with important promoter element of *HOXA10*. These results suggest that the presence of CTCF on the promoter region possibly interferes with the transcriptional machinery and thus contribute to induce transcriptional silencing of *HOXA10* in MCF-7.

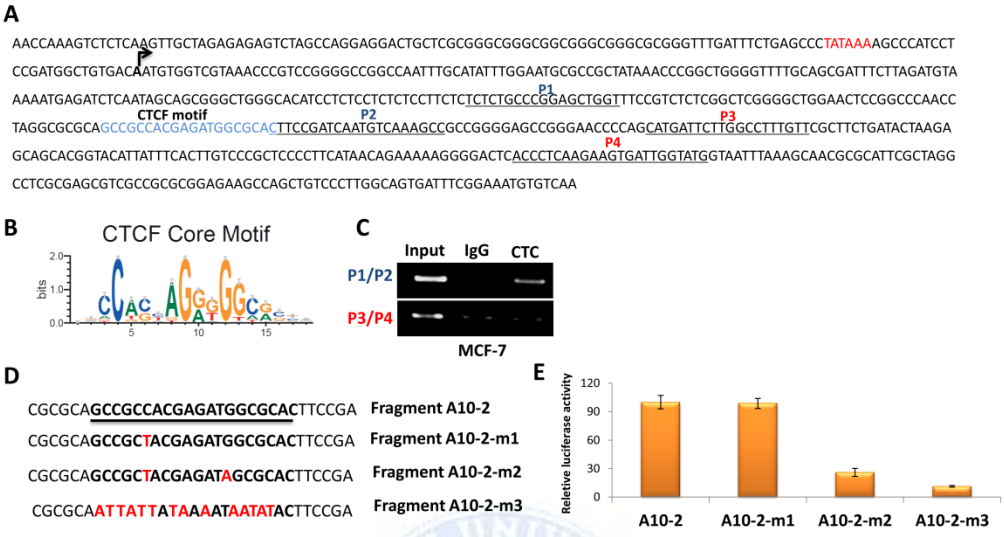


Figure 15. HOXA10 promoter analysis for CTCF core motif and promoter elements. (A) A10-2 DNA fragment sequence with predicted CTCF core binding motif and primer sites for ChIP analysis (P1 to P4). TATA box is indicated in red and transcription start site is shown as bold with arrow. (B) DNA consensus sequences for CTCF core motif shown in.[46] (C) ChIP-PCR analysis of *HOXA10* promoter region with modified ChIP protocol. Primer sets P1/P2 and P3/P4 were used. (D) DNA sequences of the A10-2 fragments with wild type and three different constructs with mutated CTCF core motifs generated for in vitro mutation assay. (E) Relative promoter activities of wild type and mutated A10-2 fragments using dual luciferase assay.

Table 4. Primers to generate A10-2-m1, A10-2-m2 and A10-2-m3 mutated plasmids according to the Figure 14

Primer	Sequence
F1	5’-AGAGGTACCAGGGTAGTGGGACAGATCT-3’
R1	5’-AGAAAGCTTTGTGGCCTCTTACCTTGAC-3’
F2 Primer for mut1	5’-GCCGCTACGAGATGGCGCACTT-3’
F2Primer for mut2	5’-TACGAGATAGCGCACTTCCGAT-3’
F2Primer for mut3	5’-ATTATTATAAAATAATATACTTCCGATCAATGTC-3’
R2Primer for mut1	5’-AAGTGCGCCATCTCGTAGCGGC-3’
R2Primer for mut2	5’-AAGTGCGCTATCTCGTAGCGGCT-3’
R2Primer for mut3	5’-TATATTATTTTATAATAATTGCGCGCCTAGGTTG-3’

5. Validation of HOXA10 regulation by CTCF in other breast cancer cell lines

In order to confirm that presence of CTCF in the promoter region of *HOXA10* induces *HOXA10* silencing, we chose two ER-positive breast cancer cell lines, BT-474 and T-47D. In these two cell lines, CTCF expression levels were similar at both mRNA (Figure 16A) and protein levels,[21] whereas *HOXA10* was differentially

expressed, i.e., BT-474 showed higher expression of *HOXA10* than T-47D (Figure 16A), which is consistent with the previous report.[44] HOXA10 in BT-474 found to be localized in cytoplasm as well as in nucleus while in T-47D cells no nuclear localization of HOXA10 was observed (Figure 16B).

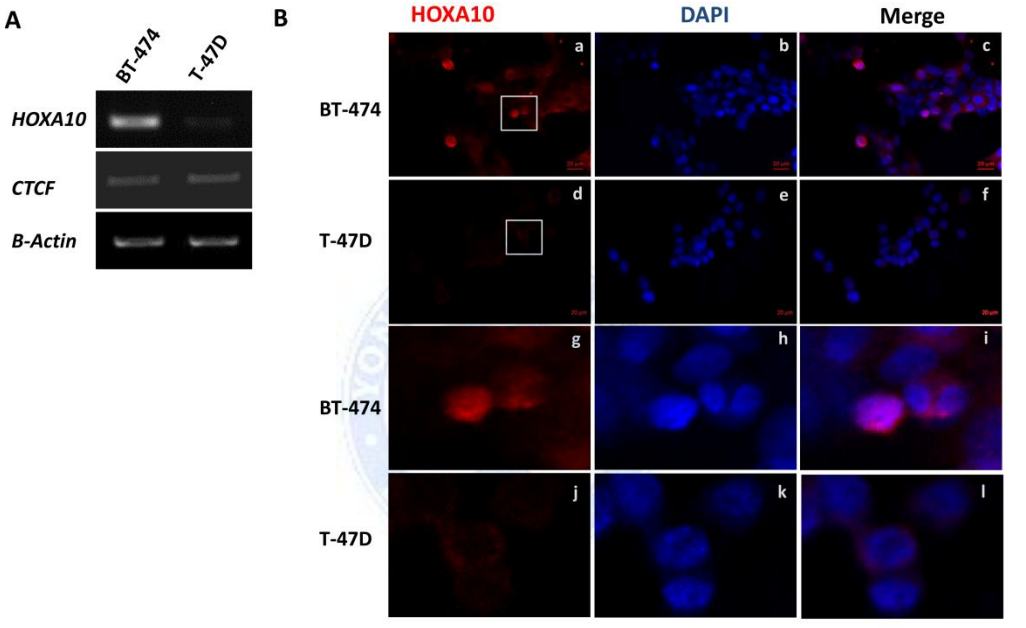


Figure 16. Differential action of CTCF on HOXA10 expression in breast cancer cell lines. (A) mRNA levels of CTCF and *HOXA10* in BT-474 and T-47D cells. (B) HOXA10 localization in T-47D and BT-474 cells. a-c and d-f show HOXA10, DAPI and overlay images in BT-474 and T-47D, respectively. g-i and j-l are the zoom images for the area highlighted by square in figure a and d, respectively.

We deduced that this effect might be caused by differential CTCF binding on *HOXA10* promoter region. ChIP-PCR analysis showed that CTCF binds preferentially on the promoter region of *HOXA10* in T-47D cells, which can be clearly attributed to the transcriptional silencing (Figure 17A). CTCF overexpression and knockdown inversely regulate *HOXA10* expression in T-47D, indicating a direct role of CTCF in *HOXA10* silencing in T-47D (Figure 17B, C). Further, overexpression of CTCF in BT-474 induce little silencing of *HOXA10* compared to T-47D cells (Figure 17D, E). These results demonstrate that *HOXA10* expression is negatively regulated by CTCF in breast cancer cells such as MCF-7 and T-47D, in which CTCF preferentially binds to the promoter region of *HOXA10*. In certain cell lines such as BT-474, CTCF does not affect *HOXA10* expression because its binding is not preferred

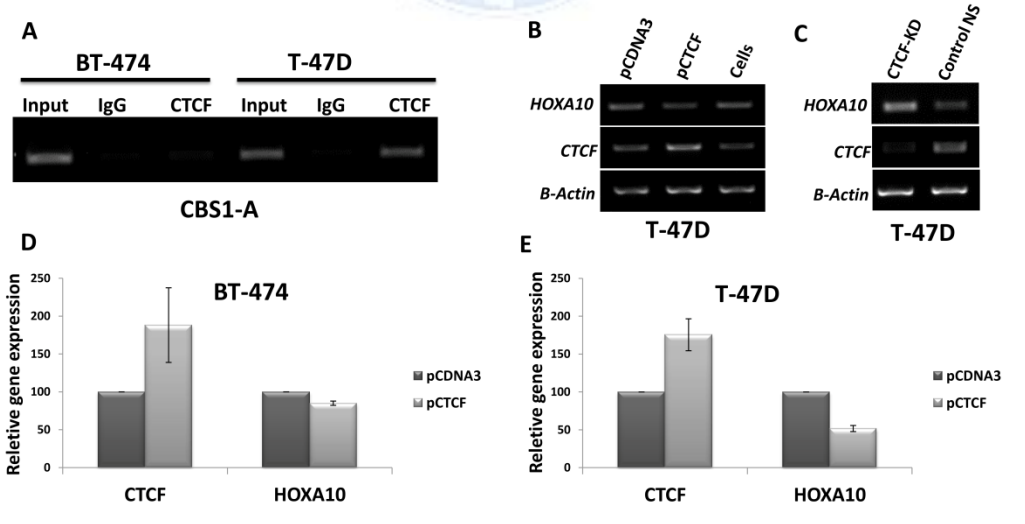


Figure 17. CTCF modulate *HOXA10* expression in T-47D cells but not in BT-474 (A) ChIP-PCR analysis of CTCF enrichment on *HOXA10* promoter region (CBS1-A) in BT-474 and T-47D cells. (B) *HOXA10* expression in control (pCDNA3) and CTCF over expressing (pCTCF) T-47D, as well as in un-treated (Cells). (C) *HOXA10* expression in CTCF knockdown (CTCF-KD) and control non-specific (Control-NS) T-47D cells. (D-E) Semi-quantitative RT-PCR of *HOXA10* expression in CTCF overexpressing BT-474 and T-47D cells. The expression of genes in controls is set as 100. n=3 and error bars are SE.

6. *CTCF* and *HOXA10* expression in breast tumor

CTCF mRNA levels in breast tumors are found to be higher as compared to its adjacent normal tissue while mRNA levels of *HOXA10* are found lower in breast tumor as compared to adjacent normal in 14 samples (Figure 18). This shows that higher levels of CTCF in breast tumor contribute in tumorigenesis by *HOXA10* silencing.

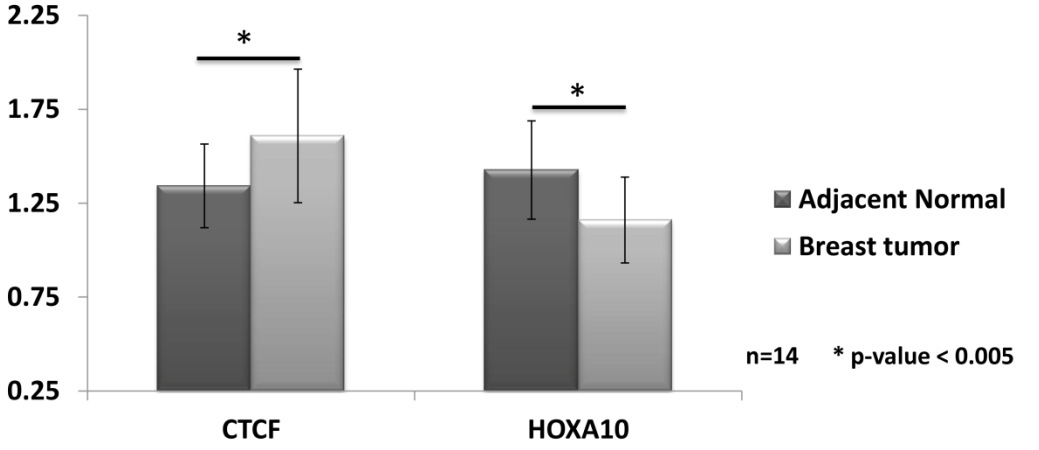


Figure 18. Analysis of *CTCF* and *HOXA10* mRNA levels in breast tumor. Semi-quantitative RT-PCR analysis of *CTCF* and *HOXA10* mRNA expression levels in 14 breast tumor and their corresponding adjacent normal samples.

7. CTCF differentially affect breast cancer cell proliferation

As CTCF is known to contribute in cancer cell survival[21] and one of the reason is by transcriptional silencing of pro-apoptotic gene Bax in breast tumor.[13] We wanted to explore how CTCF depletion affects the survival of MCF-7. We first designed a time course experiment to analyze the effect of CTCF knock down on proliferation of MCF-7 and MCF10A cells (Figure 19, Figure 20).

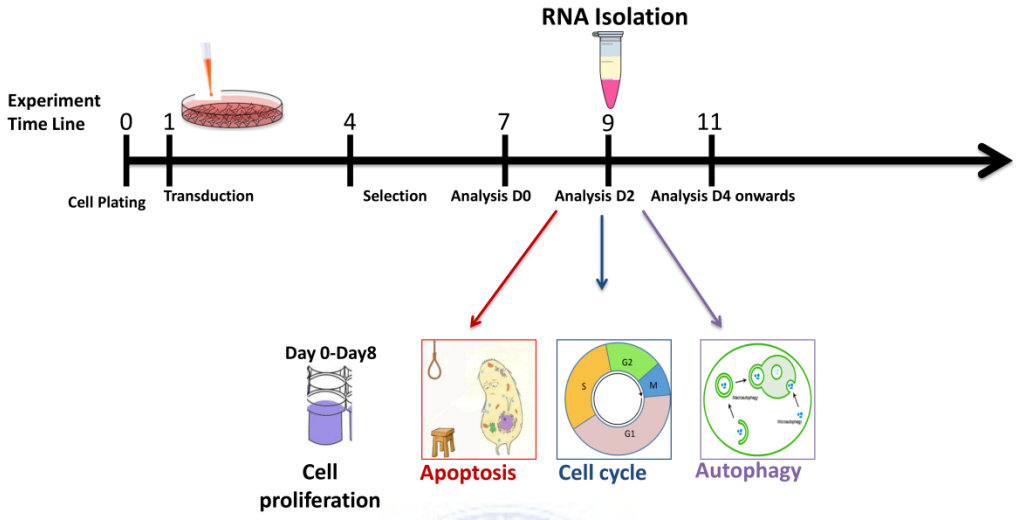


Figure 19. Experiment time line for CTCF knock down cells.

Using MTT assay we observed that depletion of CTCF affect Cellular proliferation in MCF7 cells more than in MCF10A over the period of time. This indicates a possible higher dependency of cancer cell on CTCF than normal breast epithelia cells.

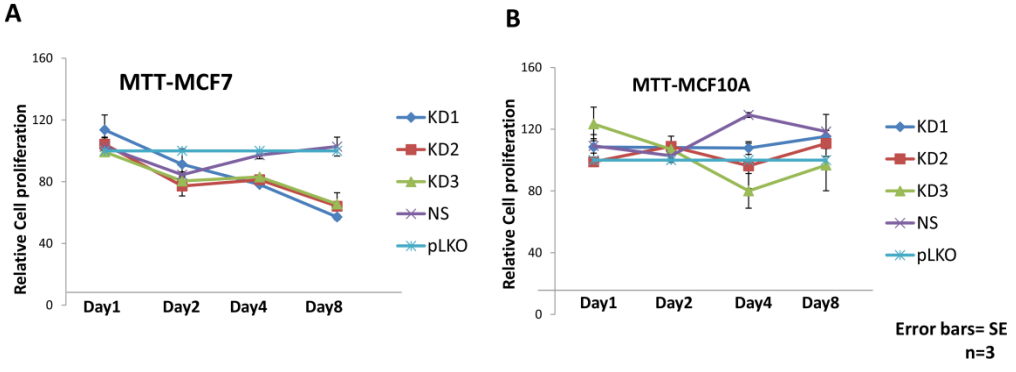


Figure 20. Effect of CTCF on cellular proliferation in MCF10A and MCF-7 cells. (A-B) Cellular proliferation using MTT assay were monitored in a time course experiment using CTCF knock down (KD1, KD2 and KD3) and control (Non-specific (NS) and empty vector (pLKO.1)) MCF-7 and MCF10A cells.

8. CTCF affects cancer cell survival and colony formation

In order to confirm that CTCF depletion is disadvantageous to the breast cancer cell lines, we developed cancer and normal cells co-culture system (MCF-7/MCF10A) and observed the effect of CTCF knock down on the two types of cells. In order to distinguish cancer cells (MCF-7) from normal breast epithelial cells (MCF10A), GFP expressing MCF-7 stable cell line clone MCF-7-GFP-H3 was generated (Figure 21A). *CTCF* mRNA levels of different GFP expressing MCF-7 clones were compared with wild type MCF-7 cells (Figure 21B). MCF-7-GFP-H3, which will be referred as MCF-7-GFP was used for co-culture. The two different cell lines can be distinguished using florescent microscopy (Figure 21C) in a co-culture system. Media was optimized to grow MCF-7-GFP/MCF10A co-culture to achieve similar growth rate (Figure 21D-E).

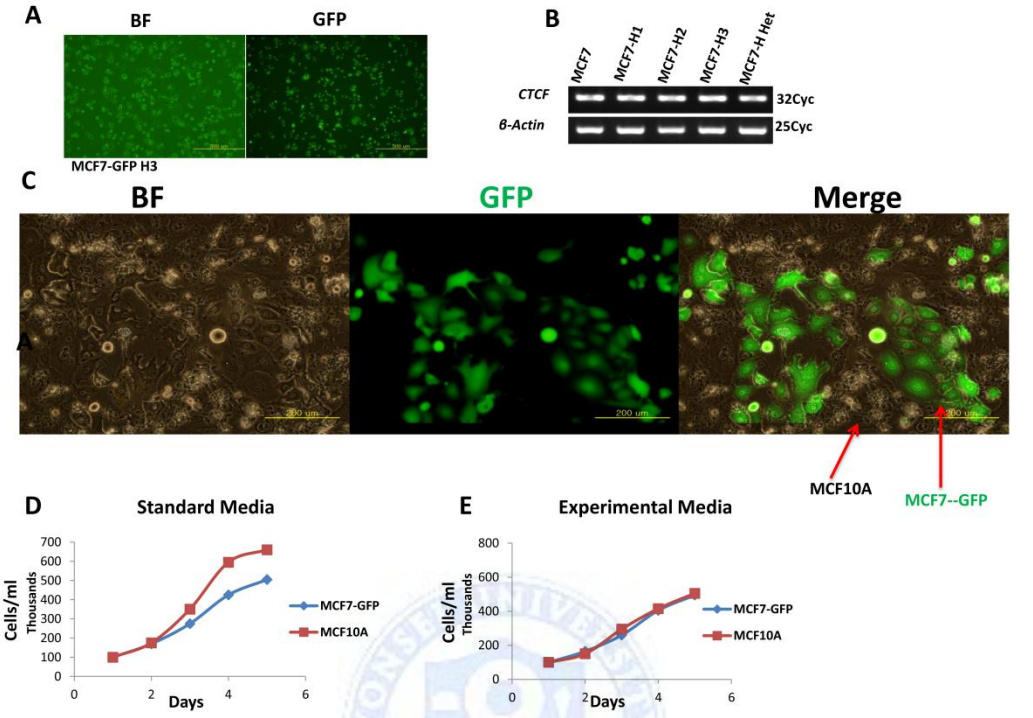


Figure 21. Optimization of MCF10A and MCF-7-GFP co culture. (A) Homogenous clone MCF-7-GFP-H3, expressing stably transfected GFP using pEGFP-N1 empty vector. (B) mRNA expression of *CTCF* in MCF-7 and GFP expressing MCF-7 clones. (C) MCF-7-GFP/MCF10A co-culture in optimized experimental media. (D-E) Growth rate of MCF-7-GFP and MCF10A cells in their standard media and in optimized experimental media.

CTCF knocked down (MCF-7-GFP) cancer and normal breast epithelial cells (MCF10A) were used to grow in co-culture with selection marker. It was observed that MCF-7-GFP cells failed to maintained their colonies and aggregates between day 4 and day 6 (Figure 19) in CTCF knock down conditions. We also failed to prepare CTCF knock down stable cell line. This observation confirms that consistent depletion of CTCF over the period of time affect cancer cell survival and colony formation.

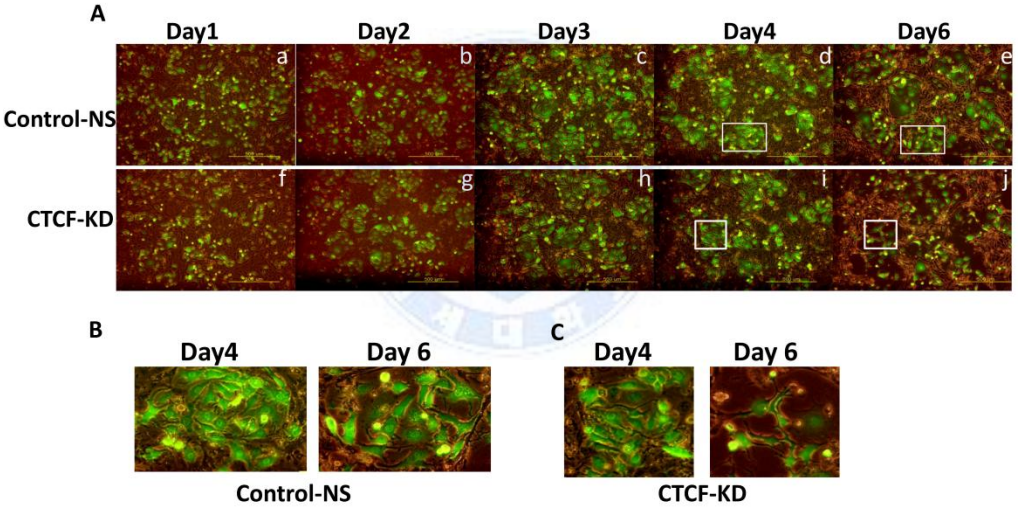


Figure 22. Growth of MCF-7/MCF10A co-culture in CTCF depleted conditions. (A) Florescent microscopy of MCF-7-GFP/MCF10A co-culture in control NS and in CTCF knock down conditions. (B-C) Zoom in images of square boxes indicated in d-e and i-j.

9. CTCF negatively regulate tumor suppressor *TP53* and *HOXC8* expression in MCF-7

Next we investigated that whether CTCF affect cancer cell survival through p53 pathway or not? We observed that mRNA levels of *TP53* and its downstream members of cascade, *p21* and *Bax* were up-regulated in CTCF-knock down cells, (Figure 23A) which can induce cell cycle arrest and apoptosis in cancer cells. Breast cancer tumor suppressor *HOXC8* is also found to be upregulated in CTCF knock down MCF-7cells.

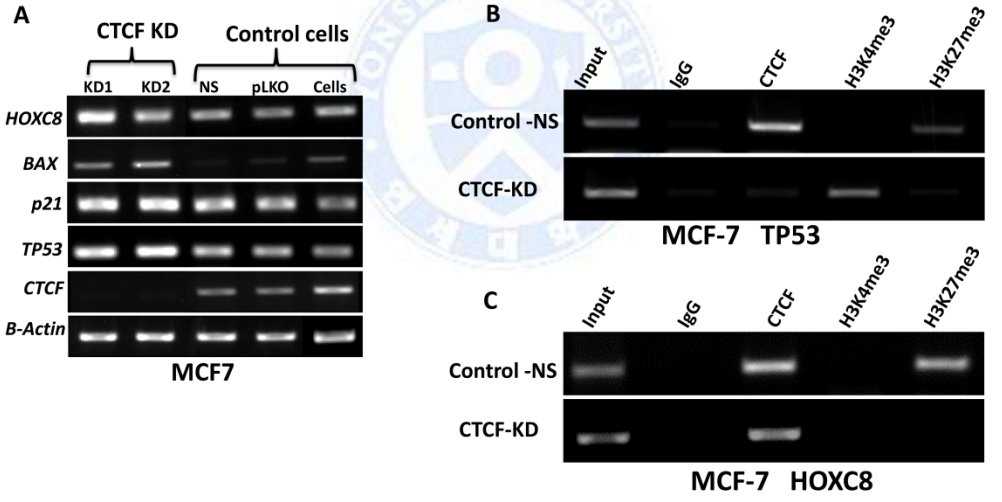


Figure 23. CTCF regulation of *TP53* and *HOXC8* in breast cancer cell line MCF-7. (A) mRNA expression of *TP53, p21, Bax* and *HOXC8* in CTCF knock down (KD1 and KD2) and control cells (NS, pLKO.1 and cells). (B-C) ChIP-PCR analysis to

observe histone modification (H3K4me3 and H3K27me3) on promoter region of *TP53* and *HOXC8* in Control NS and CTCF knock down MCF-7 cells.

TP53 and *HOXC8* harbors a CTCF binding site near their promoter region,[45] it is expected that CTCF might negatively regulates these two genes in the same was as it does in HOXA10.[13] To establish a direct transcriptional role of CTCF in regulation of *TP53* and *HOXC8,* we performed ChIP analysis to observe histone modifications on promoter region of *TP53* and *HOXC8* using our control non specific (Control-NS) and CTCF knock down MCF-7cells (CTCF-KD) (Figure 23B-C). We found that enrichment of CTCF near the promoter region of TP53 and HOXC8 is marked with repressive histone marks H3K27me3 and its depletion activate the promoter region with recruitment of active histone marks H3K4me3. Taken together we concluded that CTCF induce transcriptional silencing to *TP53* and *HOXC8* by binding to their promoter region. CTCF depletion activate promoter region of *TP53* and *HOXC8* and upregulate their transcription and transcription of their downstream genes. Our ICC data confirms that in control and CTCF knock down MCF-7 cells p53 nuclear expression is related to CTCF depleted nuclei only. (Figure 24)

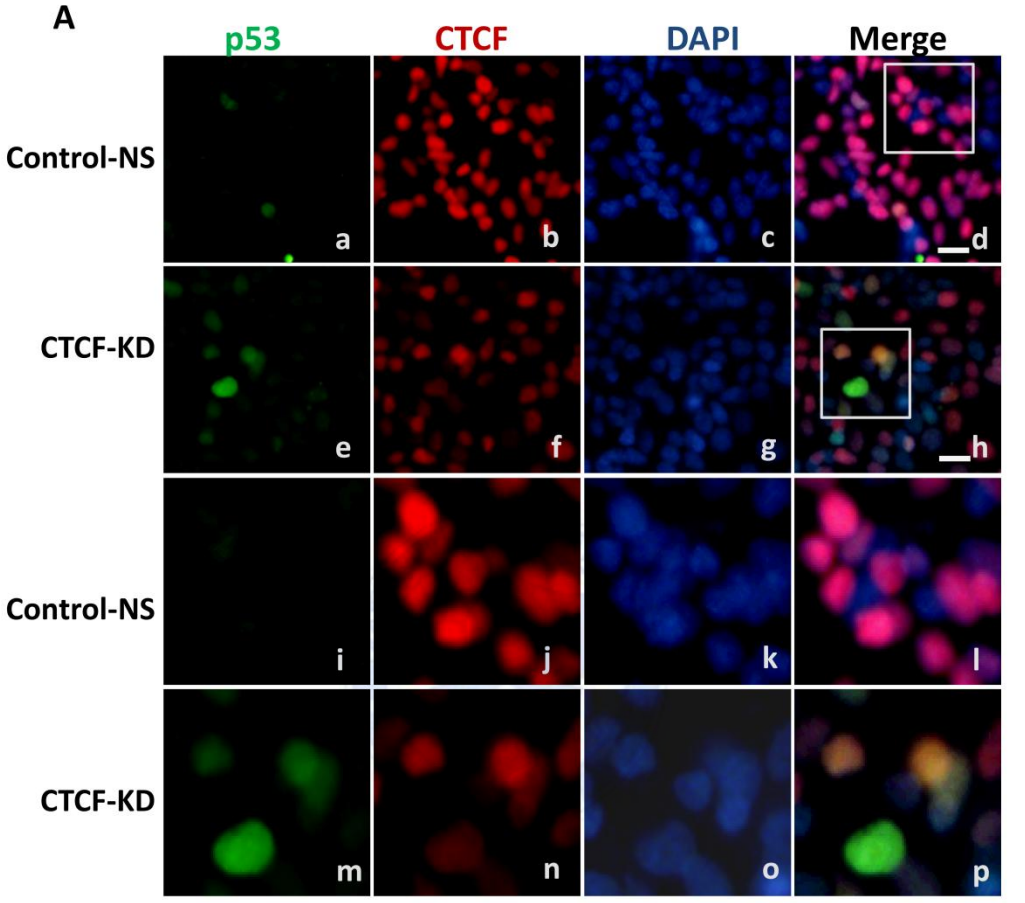


Figure 24. CTCF and p53 localization in MCF-7 cells. (A) Fluorescent microscopy images showing expression of p53 (green) and CTCF (red), DAPI (blue) and merge in control non-specific a-d and CTCF knock down e-h cells. i-l and m-p are zoom in images from the area marked in square boxes in d and h respectively. Scale bar = 20μ

m.

10. Nuclear p53 co-localize with HOXA10 in breast cancer cell lines

HOXA10 is known to activate p53,[47] and we observed negative regulation of both HOXA10 and p53 in MCF-7, next we ask whether HOXA10 and p53 expression is related to each other or not? To find the answer we did ICC experiment to see p53 and HOXA10 expression in control and CTCF knock down MCF-7 cells. We found that nuclear expression of p53 in CTCF knock down cells is strongly related to the nuclear expression of HOXA10 (Figure 25). The observed co-localization of HOXA10 and p53 established a possible link of HOXA10 with p53 nuclear localization and activation. We concluded that upregulation and nuclear localization of HOXA10 in CTCF knock down MCF-7 cells also activate or stabilize p53 possibly by nuclear import or co-localization.

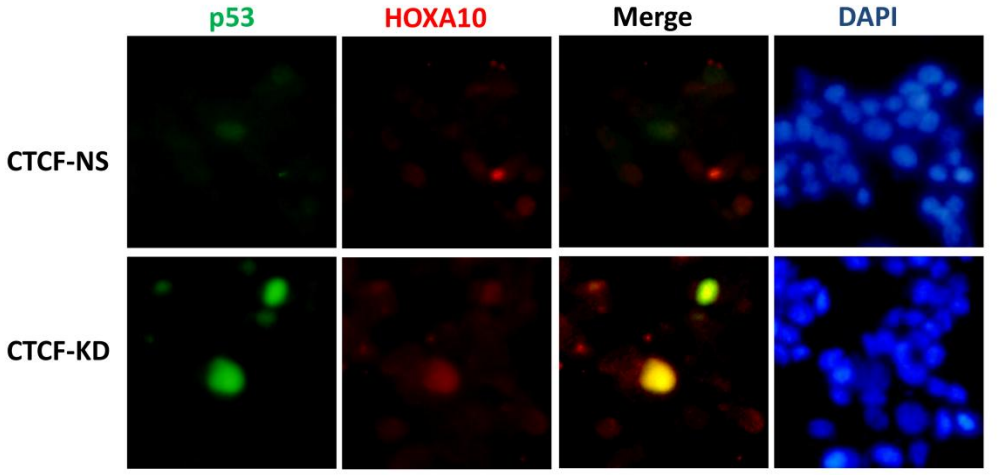


Figure 25. HOXA10 and p53 localization in MCF-7 cells. (A) Confocal microscopic images showing p53, HOXA10, DAPI and Merge of control non-specific (Control-NS) a-d and CTCF knock down (CTCF-KD) e-h MCF-7 cells.

HOXA10 and p53 co-localization was also observed in HOXA10 expressing BT-474 cells but not in T-47D cells in which the HOXA10 is silenced by CTCF.[13] (Figure 26)

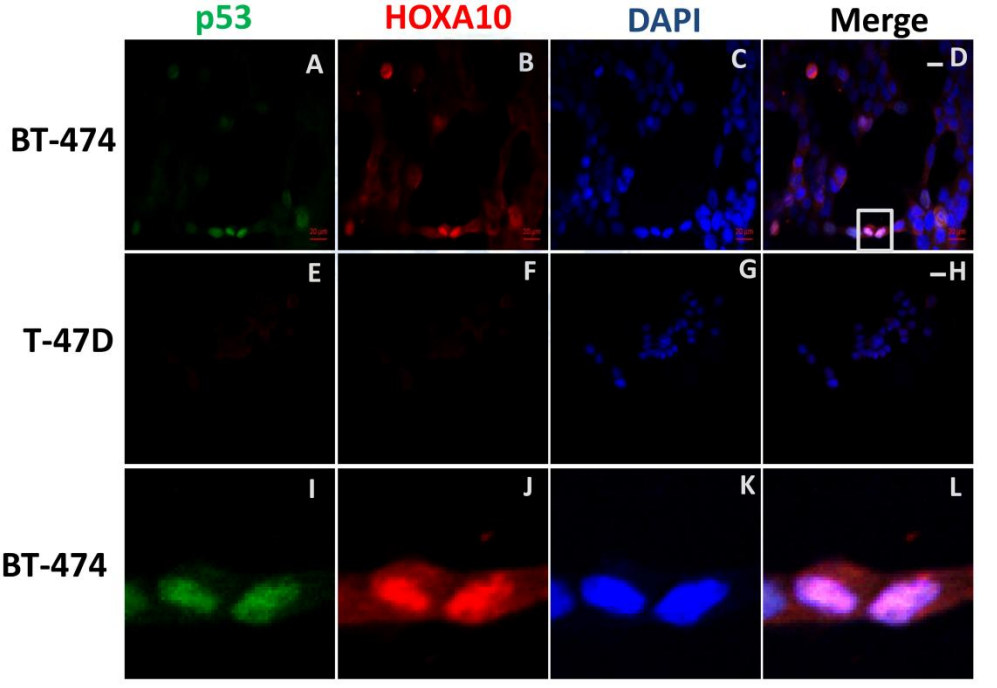


Figure 26. HOXA10 and p53 localization in BT-474 and T-47D cells. (A) Confocal

microscopy showing p53, HOXA10, DAPI and Merge of BT-474 a-d and T-47D e-h cells. i-l zoom in images of area highlighted in square box in d.

11. CTCF knock down affects cell cycle progression in MCF-7

Since CTCF depletion activates p53 cascade, therefore we next analyzed population distribution of different phases of cell cycle in control and CTCF knock down cells. Cell cycle analysis using FACS showed, CTCF knock down cells have increased population in G1 and decreased population of S phase as compared to the control non-specific cells (Figure 27). This observation indicated G1/S cell cycle arrest in CTCF knock down cells possibly as a result of upregulation of p21 (Figure23A).

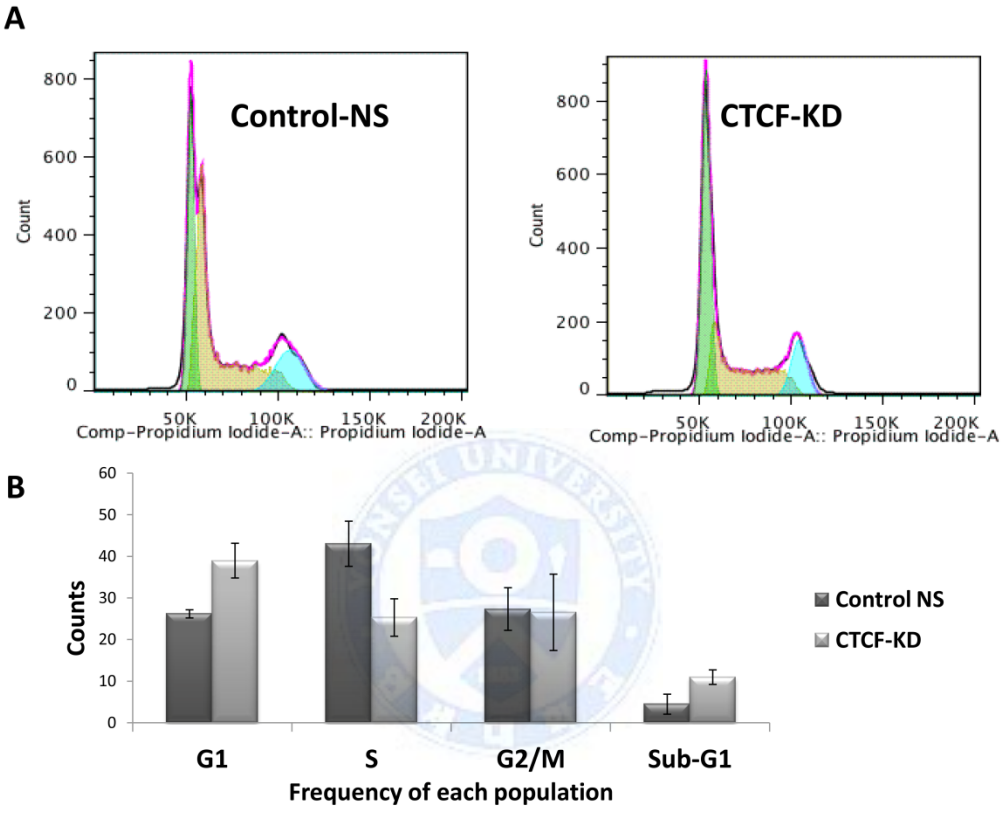


Figure 27. Fluorescence-activated cell sorting (FACS) analysis of cell cycle using PI. (A) Cell cycle distribution of control non-specific (Control-NS) and CTCF knock down (CTCF-KD) MCF-7 cells. (B) Population distribution of cell cycle phases from figure A.

12. Depleted CTCF levels in MCF-7 induce apoptosis

One possible outcome of activation of p53 cascade in CTCF knock down cells is the induction of apoptosis. To confirm that we used Annexin V and PI staining to observe early and late apoptotic cells using control and CTCF knock down cells. We found increased number of apoptotic cells in CTCF knock down conditions.

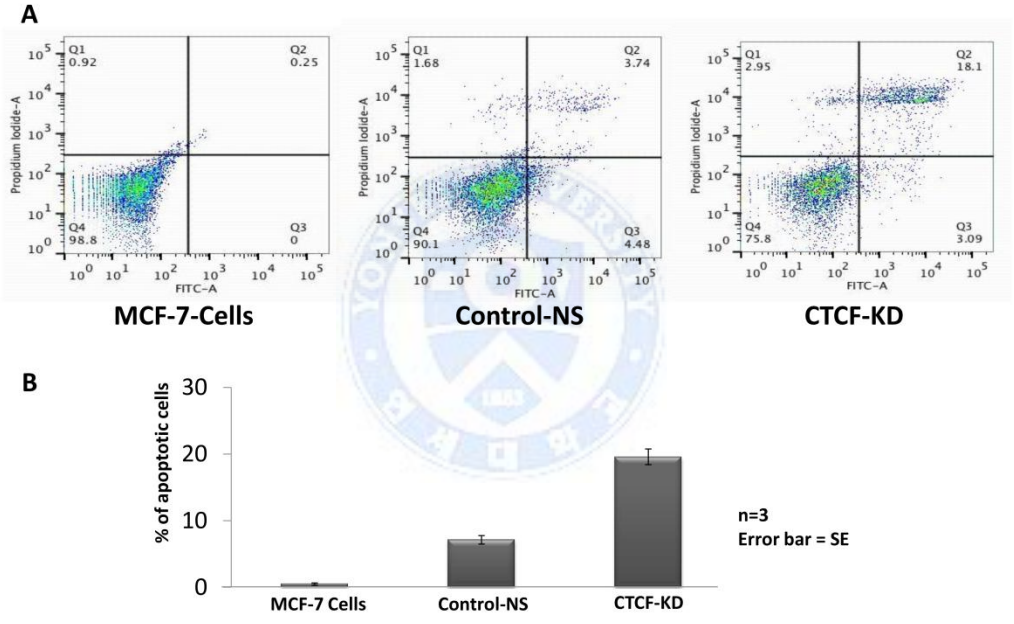


Figure 28. Fluorescence-activated cell sorting (FACS) analysis of apoptosis. (A) Representative scatter plots showing the distribution of annexin V and PI staining for control non-specific (Control-NS) and CTCF knock down (CTCF-KD) MCF-7 cells. Cells are classified as "viable" (bottom left), "early apoptotic" (bottom

right), “apoptotic” (top right), or “necrotic” (top left and right). (B) Quantitative analysis of the percentage of apoptotic by FACS analysis.

13. CTCF influence autophagy in MCF-7 cells

To analyze the effect of CTCF on autophagy, mRNA levels of two important autophagy markers p62 (SQSTM1) and ATG13 were measure and found to be up-regulated in CTCF over expressing (pCTCF) MCF-7 cells (Figure 29A). To explore further we used Lysotracker staining of lysosomes in control and CTCF knock down cells. It was observed that lysosome number decreased sharply in CTCF knock down MCF-7 cells (Figure 29B). This indicates a possibility that depletion of CTCF might block autophagy which results in reduced lysosome numbers.

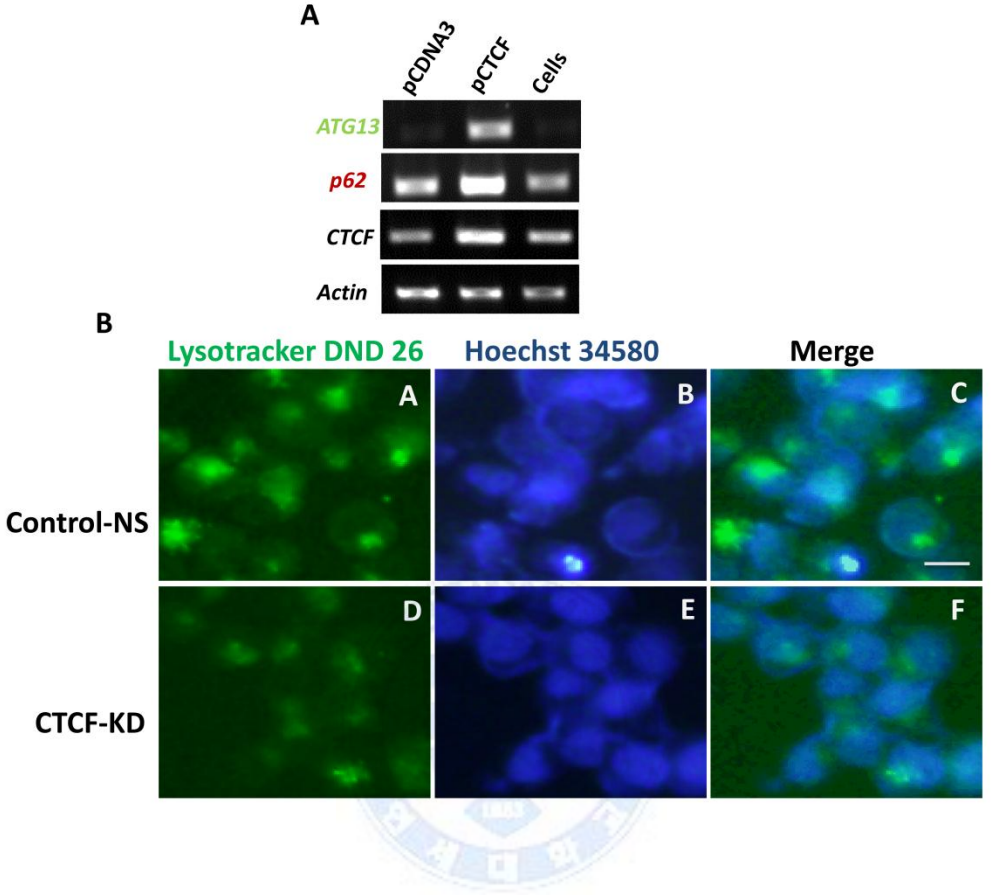


Figure 29: Analysis of autophagy. (A) mRNA levels of autophagy markers *p62* and *ATG13* in control (pCDNA3), CTCF over expressing (pCTCF) and un treated MCF-7 cells (B) Fluorescent microscopy images of MCF-7 control non-specific (Control-NS) and CTCF knock down (CTCF-KD) cells stained with lysotracker and Hoechst 34580 to show lysosomes and nucleus in live cells.

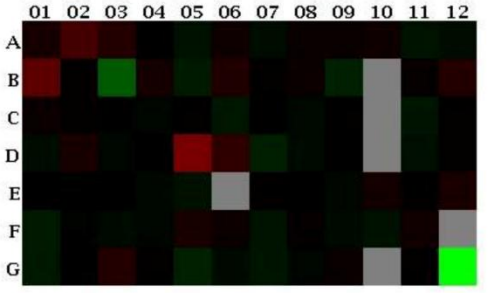
14. PCR array analysis of CTCF knock down MCF-7 cells

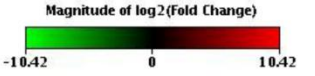
Using Human Breast Cancer RT² Profiler™ PCR Array, expression of 84 key genes commonly involved in the dysregulation of signal transduction and other normal biological processes during breast carcinogenesis were analyzed in CTCF knock down (CTCF-KD) and control non-specific (CTCF-NS) cells.This array includes genes involved in tumor classification, signal transduction, and other commonly affected pathways such as angiogenesis, adhesion, proteolysis, cell cycle, and apoptosis. It was revealed that several breast cancer markers as well as genes involved in metastasis are suppressed after CTCF knock down. The downregulation of these cancer contributors, affect the overall survival of MCF-7 cells by changing the global transcriptome in favour of reduced progression and tumorigenesis.

Table 5. Functional gene grouping for PCR array analysis

Class	Members
Tumor Classification Markers	Luminal A-C: ESR1 (ERa), FOXA1, GATA3, KRT8, KRT18, SLC39A6, TFF3, XBP1. HER2-like: ERBB2 (HER2), GRB7.
	Basal-like / Triple Negative: BIRC5, EGFR, KRT5, NOTCH1.
	Metastasis to Lung: ID1, MMP2 (Gelatinase A), PTGS2 (COX2).
Signal Transduction	Steroid Receptor-Mediated: AR, BRCA1, CCNE1, CTNNB1, ESR1 (ERa), ESR2 (ERß), IGF1, KRT19, PGR, RB1
	Hedgehog: BCL2, CCND1, GLI1, SNAI2
	Glucocorticoid: IGFBP3, NME1 (NM23A), NR3C1 (GRL)
	Classical WNT: APC, CCND1, CTNNB1, SFRP1
	PI3K/AKT: AKT1, ERBB2 (HER2), IGF1, IGF1R, PTEN.
	NOTCH: BIRC5, NOTCH1.
	MAPK: MAPK1 (ERK2), MAPK3 (ERK1), MAPK8 (JNK1), TP73
EMT	CTNNB1, NOTCH1, SRC, TGFB1, TWIST1
Angiogenesis	CDH13, CTNNB1, EGF, ERBB2 (HER2), ID1, IL6, JUN, NOTCH1, PLAU (uPA), PTEN, SERPINE1 (PAI-1), SLIT2, THBS1, VEGFA
Adhesion	ADAM23, APC, BCL2, CDH1 (E-Cadherin), CDH13, CDKN2A (p16INK4), CSF1 (MCSF), CTNNB1, EGFR, ERBB2 (HER2), PTEN, TGFB1, THBS1
Proteolysis	ADAM23, CST6, CTSD, MMP2 (Gelatinase A), MMP9 (Gelatinase B), PLAU (uPA), PYCARD (ASC).
Apoptosis	AKT1, APC, BAD, BCL2, CDH1 (E-CADHERIN), CDH1 (E-CADHERIN)3, CDKN1A, P21CIP1/WAF1), CDKN 2A (p16INK4), GSTP1, IGF1, IL6, JUN, MUC1, NME1 (NM23A), RARB, SFN (14-3-3s), SFRP1, TP53, TP73, TWIST1
Cell Cycle	APC, BCL2, CCNA1, CCND1, CCND2, CCNE1, CDK2, CDKN1A (p21CIP1/WAF1), CDKN1C (p57KIP2), CDKN2A (p16INK4), JUN, MKI67, MYC, PTEN, RASSF1, RB1, SFN (14-3-3s), TP53
Xenobiotic Transport	ABCB1 (MDR-1), ABCG2 (BCRP).
Transcription Factors	AR, CTNNB1, ESR1 (ERa), ESR2 (ERß), FOXA1, GATA3, HIC1, JUN, MYC, NOTCH1, NR3C1 (GRL), PGR, PRDM2 (RIZ1), RARB, RB1, TP53, TP73, XBP1

A





B

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	ABCB1 **2.02** B	ABCG2 **7.01** OKAY	ADAM23 **2.80** OKAY	AKT1 **-1.01** OKAY	APC **-1.70** OKAY	AR **1.61** OKAY	ATM **-1.48** OKAY	BAD **1.33** OKAY	BCL2 **1.29** B	BIRC5 **1.59** OKAY	BRCA1 **-1.75** OKAY	BRCA2 **-1.51** OKAY
B	CCNA1 **14.21** B	CCND1 **1.15** OKAY	CCND2 **-13.06** B	CCNE1 **1.85** OKAY	CDH1 **-2.24** OKAY	CDH13 **2.59** B	CDK2 **1.11** OKAY	CDKN1A **1.41** OKAY	CDKN1C **-2.67** OKAY	CDKN2A **1.10** C	CSF1 **1.22** OKAY	CST6 **2.80** B
C	CTNNB1 **1.39** OKAY	CTSD **1.13** OKAY	EGF **-1.07** B	EGFR **-1.18** OKAY	ERBB2 **1.09** OKAY	ESR1 **-1.93** OKAY	ESR2 **1.01** B	FOXA1 **-1.31** OKAY	GATA3 **1.04** OKAY	GLI1 **1.10** C	GRB7 **-1.88** OKAY	GSTP1 **1.13** OKAY
D	HIC1 **-1.46** B	ID1 **2.07** OKAY	IGF1 **-1.19** B	IGF1R **-1.02** OKAY	IGFBP3 **29.79** OKAY	IL6 **3.86** A	JUN **-2.38** OKAY	KRT18 **-1.37** OKAY	KRT19 **-1.00** OKAY	KRT5 **1.10** C	KRT8 **-1.53** OKAY	MAPK1 **1.14** OKAY
E	MAPK3 **1.07** OKAY	MAPK8 **-1.04** OKAY	MGMT **1.03** OKAY	MKI67 **-1.26** OKAY	MLH1 **-1.64** OKAY	MMP2 **1.10** C	MMP9 **1.15** OKAY	MUC1 **-1.01** OKAY	MYC **-1.23** OKAY	NME1 **1.74** OKAY	NOTCH1 **1.07** OKAY	NR3C1 **2.16** OKAY
F	PGR **-2.24** OKAY	PLAU **-1.13** B	PRDM2 **-1.37** OKAY	PTEN **-1.22** OKAY	PTGS2 **2.05** B	PYCARD **1.43** OKAY	RARB **-1.72** OKAY	RASSF1 **1.17** OKAY	RB1 **-1.44** OKAY	SERPINE1 **-1.70** A	SFN **1.63** OKAY	SFRP1 **1.10** C
G	SLC39A6 **-2.15** OKAY	SLIT2 **1.01** B	SNAI2 **2.81** OKAY	SRC **1.03** OKAY	TFF3 **-2.69** OKAY	TGFB1 **-1.41** OKAY	THBS1 **-1.83** OKAY	TP53 **-1.15** OKAY	TP73 **1.38** A	TWIST1 **1.10** C	VEGFA **1.05** OKAY	XBP1 **-1370.17** OKAY

Figure 30. Human breast cancer PCR array profile. (A) Heat map showing change in expression of mRNA levels in CTCF knock down vs control cells. (B) Plate labeling with gene annotations and fold change of expression.

15. Significance of HOXA10 in activation of p53 in breast cancer patients

HOXA10 is known to interact directly with *TP53* (Figure 31), this interaction will activate p53 cascade to induce cell cycle arrest and apoptosis provided that a functional p53 is available in the system. In order to investigate that HOXA10 expression is significantly associated with breast cancer with reference to p53, we used Kaplan Meier survival analysis of breast cancer patients. It was revealed that

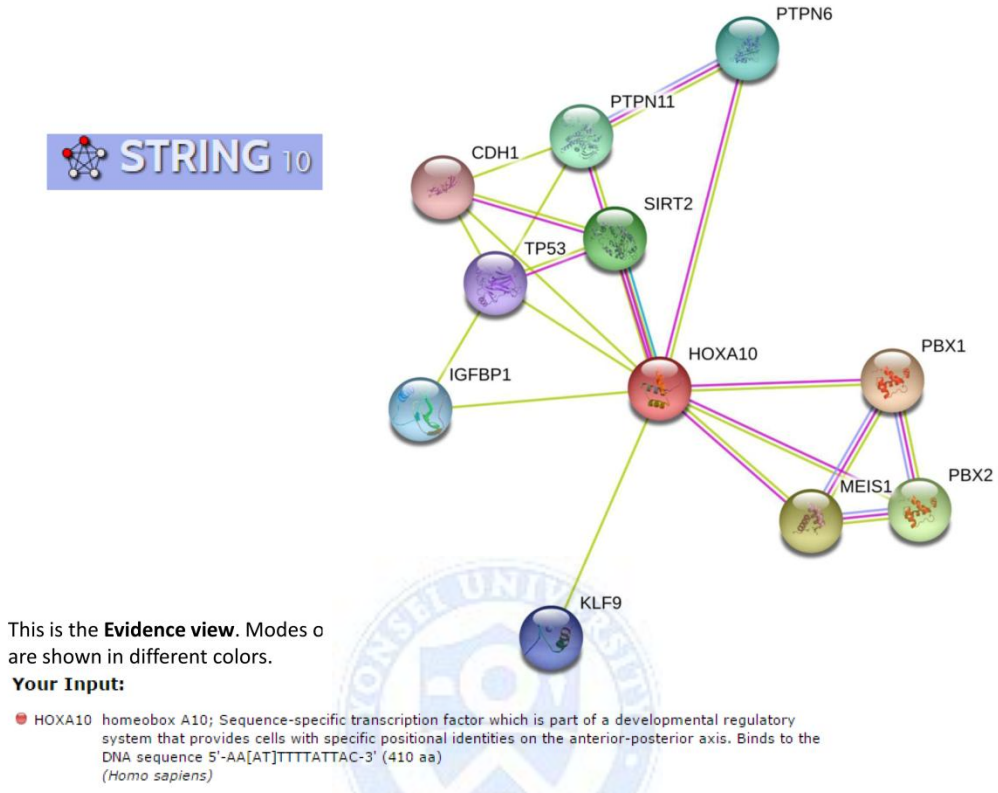


Figure 31. STRING - Known and Predicted Protein-Protein Interactions of HOXA10 with p53.

higher expression of HOXA10 does not affect the overall survival (OS) and distant metastasis free survival (DMSF) of breast cancer patients (Figure 32A, B). On the other hand patients with higher HOXA10 have significantly higher probability of OS and DMFS if they carry a wild type copy of p53 (Figure 32 C, D). We concluded from this observation that HOXA10 asserts its tumor suppressor role via activation of p53 in breast cancer patients.

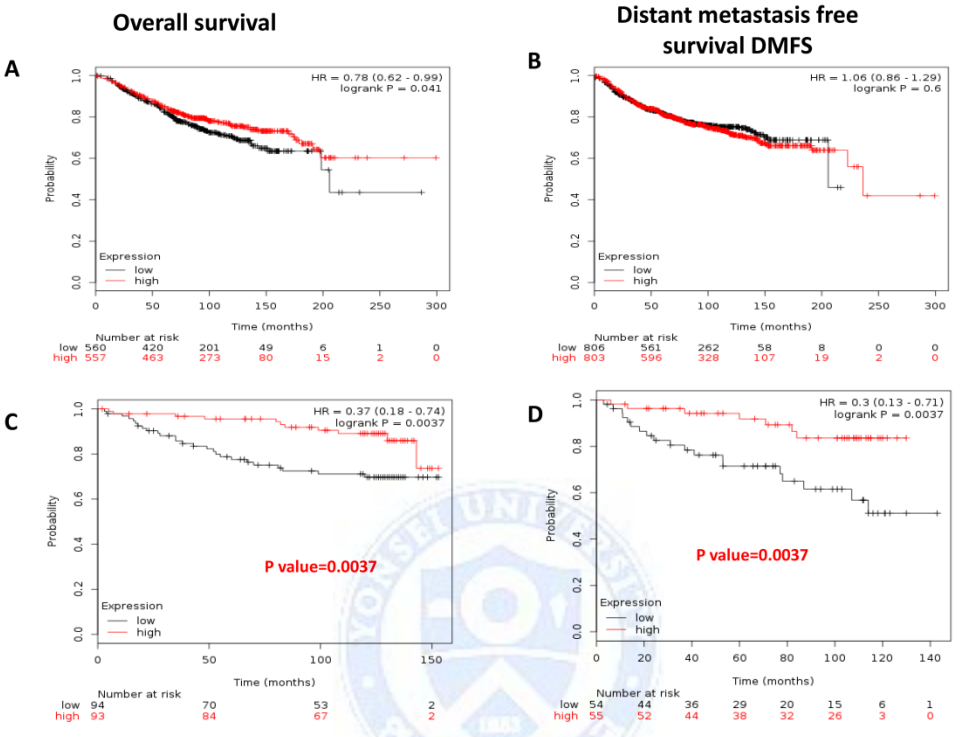


Figure 32: Kaplan Meier survival analysis of breast cancer patients. (A-B) Overall survival and distant metastasis free survival of breast cancer patients with high and low expression of HOXA10 in all patients. (C-D) Overall survival and distant metastasis free survival of breast cancer patients with high and low expression of HOXA10 in patients with wild type p53.

IV. DISCUSSION

In this study, we provide experimental evidence to explain how CTCF contribute in breast cancer tumorigenesis. We identified *HOXA10* as one of the directly regulated tumor suppressor gene by CTCF at transcriptional level in breast cancer cell lines. Our dual luciferase reporter assay data defined novel promoter region of *HOXA10*, and moreover, ChIP analysis established the inverse relationship between CTCF enrichment on the promoter region and transcriptional activity of *HOXA10* gene. Depletion of CTCF was marked with decreased repressive histone marks H3K27me3 on *HOXA10* promoter region in MCF-7 cells and differential CTCF binding inversely related to the expression levels of *HOXA10* in BT-474 and T-47D cells. *HOXA10* has three exons and two transcript variants, transcript variant 1 (originated from exon 2 and exon 3) and 2 (originated from exon 1 and exon 3). The functional protein is encoded by transcript variant 1. Our work provide evidence that *HOXA10* lacks promoter activity immediately upstream of its protein coding exon 2, instead the *HOXA10* locus showed promoter activity 5.3-6.1 kb upstream of its start codon within the non-coding exon 1. Chromatin interaction analysis with paired end tag sequencing (ChIA-PET) have revealed that extensive RNA POL2 based chromatin interaction exists between 3' of exon 1 and 5' of exon 2 region of genomic DNA on *HOXA10* locus.[48] The presence of RNA POL2 on *HOXA10* promoter region creates active chromatin confirmation of *HOXA10* locus with promoter region that lies in the three

dimensional (3D) neighborhood of protein coding exon 2 of *HOXA10*. CTCF may oppose this active state in MCF-7 cells by not allowing the recruitment RNA POL2.

In most cases the CTCF binding is regulated by CpG methylation,[43] therefore, it is highly likely that methylation status of CpGs present the promoter region of *HOXA10* in breast cancer cell lines defines the CTCF enrichment and *HOXA10* expression. *HOXA10* expression in T-47D cells can be modulated with CTCF, while *HOXA10* expression seems to be independent of CTCF in BT-474 cells which have little enrichment of CTCF on *HOXA10* promoter (Figure 17). This effect appeared possibly due to differential methylation pattern, which make CTCF binding on *HOXA10* promoter to be more favorable in T-47D than in BT-474 cells.

We identified a CTCF core motif, based on previously reported consensus sequence,[46] within *HOXA10* promoter region. It was demonstrated previously that CTCF protein interacts with POLR2A,[49] which is enough to interplay with transcription machinery. Using in vitro mutation assay we found that CTCF core motif within the *HOXA10* promoter region is flanked with an important promoter element (Figure 15). This promoter element is no longer available to take part in transcriptional activity as long as it is occupied with CTCF. The presence of CTCF near the promoter region of *HOXA10* induces silencing effect not only by interfering transcriptional machinery but also by maintaining repressive chromatin state. One possibility is that CTCF-POLR2A interaction can induce RNA poll II stalling to reduce transcription of HOXA10. CTCF based transcriptional silencing is not limited to *HOXA10* in this

study but we also found that *HOXC8* and *TP53* are also regulated by CTCF in the same way.

In BRCA mutant breast cancer patients, several *HOX* genes including *HOXA10* are frequently silenced by hypermethylation.[37] HOXA10 expression regulates p53 in breast cancer cells[34] and its reduced expression contributes towards invasion and metastasis.[50] Higher levels of CTCF gives survival advantage to breast cancer cells by conferring resistance towards apoptosis as a result of epigenetic silencing of Bax[13, 21] as well as tumor suppressors *HOXA10,HOXC8* and *TP53* in the same way. In this study we present a novel silencing mechanism of *HOXA10* in breast cancer cell lines by CTCF occupancy on the core promoter region of *HOXA10*.[51] This is different from general mechanism of methylation-dependent gene silencing which can inactivate the promoter region of *HOXA10* for all transcription factors.

Inverse transcriptional regulation of target genes by CTCF, harboring a CTCF binding site near promoter region, works similarly and can be rationalized as in case of *Bax* and *HOXA10*. The CTCF presence near promoter region not only maintains inactive local chromatin state but also interferes with transcriptional machinery to induce silencing. Identification of other molecules which can be negatively regulated by CTCF will help to understand how a single factor CTCF affects the overall transcriptome of breast cancer cells.

CTCF mediated silencing of tumor suppressor like *HOXA10*, *HOXC8* and *TP53* downplay the function of a key anticancer pathway in breast cancer cells. Depletion

of CTCF in breast cancer cells activates p53 cascade directly or via HOXA10 and induce cell cycle arrest as well as apoptosis. *HOXA10* upregulation increase nuclear HOXA10 which co-localize with p53 and might involve its nuclear import or stabilization. Since MCF-7 carries a wild type p53 therefore a cancer phenotype of MCF-7 is strongly related to either, transcriptional downregulation of p53 gene or inactivation of p53 protein by MDM2, the main ubiquitin ligase of p53. In the context of MCF-7, CTCF can serve this purpose by transcription downregulation of p53 as well as *HOXA10*. As a result of cellular stress, p53 is stabilized and rapidly accumulates in the nucleus to serve its function.[52] We showed that nuclear localization of p53 in MCF-7 cells is inhibited by the CTCF and associated with nuclear HOXA10. Nuclear HOXA10 might contribute in p53 nuclear localization to trigger cell's internal defense mechanism in MCF-7 cells. Identification and restoration of tumor suppressors which are inhibited by CTCF can be achieved by targeted genome engineering of CTCF binding sites. Reactivation of cellular resources to cope oncogenic transformation could provide an additional choice of treatment in the ongoing struggle against cancer.

V. CONCLUSION

CTCF inversely regulates *HOXA10*, *HOXC8* and *TP53* in breast cancer cells. HOXA10 promoter was identified 5.3-6.1 kb upstream of the protein coding region (Figure 33). CTCF binding to the promoter region maintains inactive local chromatin state at *HOXA10* promoter and induce transcriptional silencing. In addition to that CTCF presence on *HOXA10* promoter interferes with transcriptional machinery by flanking important promoter element. Depletion of CTCF in breast cancer cells results in activation of *HOXA10* promoter region and increased transcription.[13]

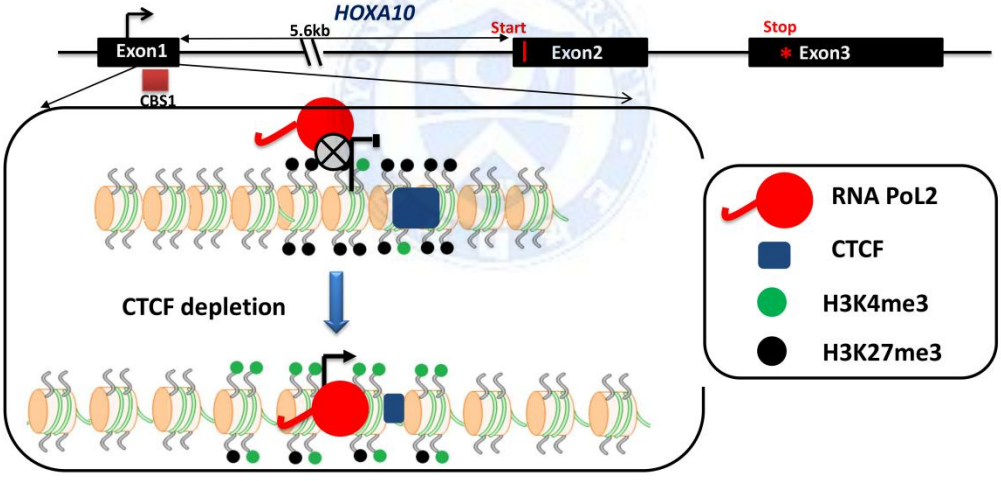


Figure 33. Mechanism of HOXA10 regulation.

CTCF confers transcriptional silencing of p53 cascade directly or through HOXA10 to contribute in breast cancer tumorigenesis. CTCF depletion activates *TP53* and

increased nuclear HOXA10 which co-localize with p53. The activation of p53 induces cell cycle arrest and apoptosis and suppresses cancer cell progression and survival.

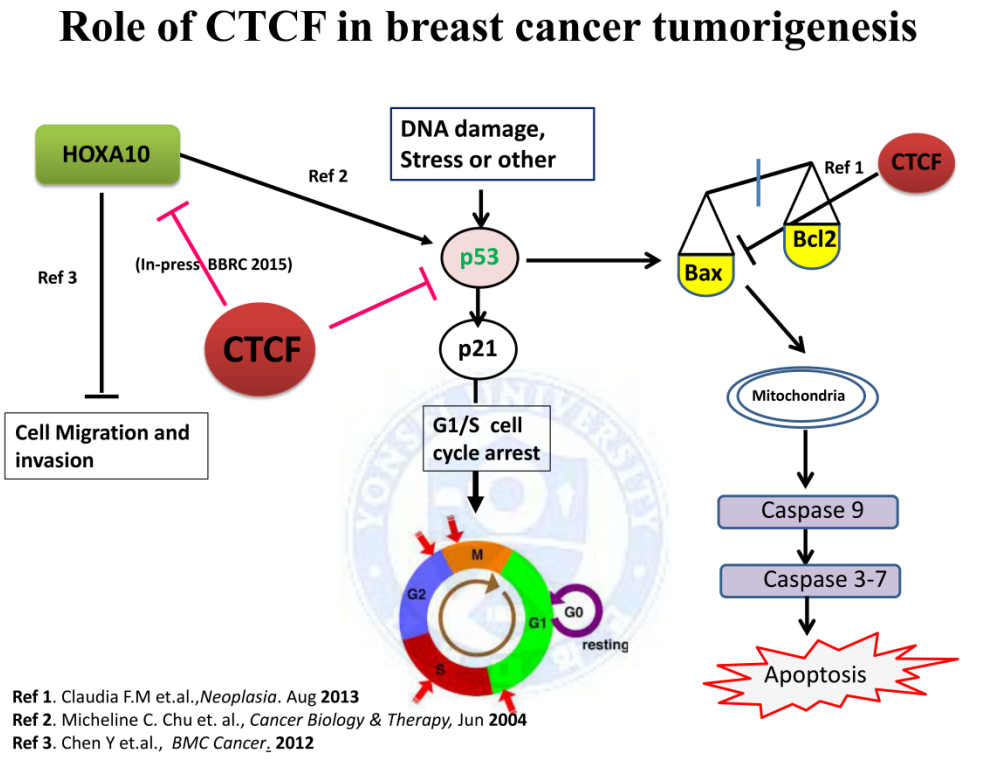


Figure 34. CTCF based inactivation of p53 cascade.

REFERENCES

1. Phillips JE, Corces VG. CTCF: master weaver of the genome. Cell. 2009 Jun 26;137(7):1194-211.
2. Filippova GN, Fagerlie S, Klenova EM, Myers C, Dehner Y, Goodwin G, et al. An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-myc oncogenes. Molecular and cellular biology. 1996 Jun;16(6):2802-13.
3. Vostrov AA, Taheny MJ, Quitschke WW. A region to the N-terminal side of the CTCF zinc finger domain is essential for activating transcription from the amyloid precursor protein promoter. The Journal of biological chemistry. 2002 Jan 11;277(2):1619-27.
4. Majumder P, Gomez JA, Chadwick BP, Boss JM. The insulator factor CTCF controls MHC class II gene expression and is required for the formation of long-distance chromatin interactions. The Journal of experimental medicine. 2008 Apr 14;205(4):785-98.
5. Lutz M, Burke LJ, Barreto G, Goeman F, Greb H, Arnold R, et al. Transcriptional repression by the insulator protein CTCF involves histone deacetylases. Nucleic acids research. 2000 Apr 15;28(8):1707-13.
6. Heintzman ND, Hon GC, Hawkins RD, Kheradpour P, Stark A, Harp LF, et al. Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature. 2009 May 7;459(7243):108-12.
7. Splinter E, Heath H, Kooren J, Palstra RJ, Klous P, Grosveld F, et al. CTCF mediates long-range chromatin looping and local histone modification in the beta-globin locus. Genes & development. 2006 Sep 1;20(17):2349-54.

8. Gombert WM, Krumm A. Targeted deletion of multiple CTCF-binding elements in the human C-MYC gene reveals a requirement for CTCF in C-MYC expression. PloS one. 2009;4(7):e6109.
9. Williams A, Flavell RA. The role of CTCF in regulating nuclear organization. The Journal of experimental medicine. 2008 Apr 14;205(4):747-50.
10. Klenova EM, Morse HC, 3rd, Ohlsson R, Lobanenkov VV. The novel BORIS + CTCF gene family is uniquely involved in the epigenetics of normal biology and cancer. Seminars in cancer biology. 2002 Oct;12(5):399-414.
11. Klenova EM, Nicolas RH, Paterson HF, Carne AF, Heath CM, Goodwin GH, et al. CTCF, a conserved nuclear factor required for optimal transcriptional activity of the chicken c-myc gene, is an 11-Zn-finger protein differentially expressed in multiple forms. Molecular and cellular biology. 1993 Dec;13(12):7612-24.
12. Renaud S, Loukinov D, Bosman FT, Lobanenkov V, Benhattar J. CTCF binds the proximal exonic region of hTERT and inhibits its transcription. Nucleic acids research. 2005;33(21):6850-60.
13. Mendez-Catala CF, Gretton S, Vostrov A, Pugacheva E, Farrar D, Ito Y, et al. A novel mechanism for CTCF in the epigenetic regulation of Bax in breast cancer cells. Neoplasia. 2013 Aug;15(8):898-912.
14. Holwerda SJ, de Laat W. CTCF: the protein, the binding partners, the binding sites and their chromatin loops. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2013;368(1620):20120369.
15. Filippova GN, Qi CF, Ulmer JE, Moore JM, Ward MD, Hu YJ, et al. Tumor-associated zinc finger mutations in the CTCF transcription factor selectively alter tts DNA-binding specificity. Cancer research. 2002 Jan 1;62(1):48-52.
16. Ohlsson R, Lobanenkov V, Klenova E. Does CTCF mediate between nuclear organization and gene expression? BioEssays : news and reviews in molecular, cellular and developmental biology. 2010 Jan;32(1):37-50.
17. Fiorentino FP, Giordano A. The tumor suppressor role of CTCF. Journal of cellular physiology. 2012 Feb;227(2):479-92.
18. Recillas-Targa F, De La Rosa-Velazquez IA, Soto-Reyes E, Benitez-Bribiesca L. Epigenetic boundaries of tumour suppressor gene promoters: the CTCF

connection and its role in carcinogenesis. Journal of cellular and molecular medicine. 2006 Jul-Sep;10(3):554-68.

19. Saldana-Meyer R, Gonzalez-Buendia E, Guerrero G, Narendra V, Bonasio R, Recillas-Targa F, et al. CTCF regulates the human p53 gene through direct interaction with its natural antisense transcript, Wrap53. Genes & development. 2014 Apr 1;28(7):723-34.

20. Rayess H, Wang MB, Srivatsan ES. Cellular senescence and tumor suppressor gene p16. International journal of cancer Journal international du cancer. 2012 Apr 15;130(8):1715-25.

21. Docquier F, Farrar D, D'Arcy V, Chernukhin I, Robinson AF, Loukinov D, et al. Heightened expression of CTCF in breast cancer cells is associated with resistance to apoptosis. Cancer research. 2005 Jun 15;65(12):5112-22.

22. Svingen T, Tonissen KF. Hox transcription factors and their elusive mammalian gene targets. Heredity. 2006 Aug;97(2):88-96.

23. Garcia-Fernandez J. The genesis and evolution of homeobox gene clusters. Nature reviews Genetics. 2005 Dec;6(12):881-92.

24. Takahashi Y, Hamada J, Murakawa K, Takada M, Tada M, Nogami I, et al. Expression profiles of 39 HOX genes in normal human adult organs and anaplastic thyroid cancer cell lines by quantitative real-time RT-PCR system. Experimental cell research. 2004 Feb 1;293(1):144-53.

25. McGinnis W, Krumlauf R. Homeobox genes and axial patterning. Cell. 1992 Jan 24;68(2):283-302.

26. Abate-Shen C. Deregulated homeobox gene expression in cancer: cause or consequence? Nature reviews Cancer. 2002 Oct;2(10):777-85.

27. Chen HS, Martin KA, Lu F, Lupey LN, Mueller JM, Lieberman PM, et al. Epigenetic deregulation of the LMP1/LMP2 locus of Epstein-Barr virus by mutation of a single CTCF-cohesin binding site. Journal of virology. 2014 Feb;88(3):1703-13.

28. Zhang X, Zhu T, Chen Y, Mertani HC, Lee KO, Lobie PE. Human growth hormone-regulated HOXA1 is a human mammary epithelial oncogene. The Journal of biological chemistry. 2003 Feb 28;278(9):7580-90.

29. Boimel PJ, Cruz C, Segall JE. A functional in vivo screen for regulators of

tumor progression identifies HOXB2 as a regulator of tumor growth in breast cancer. Genomics. 2011 Sep;98(3):164-72.

30. Raman V, Martensen SA, Reisman D, Evron E, Odenwald WF, Jaffee E, et al. Compromised HOXA5 function can limit p53 expression in human breast tumours. Nature. 2000 Jun 22;405(6789):974-8.

31. Wu X, Chen H, Parker B, Rubin E, Zhu T, Lee JS, et al. HOXB7, a homeodomain protein, is overexpressed in breast cancer and confers epithelial-mesenchymal transition. Cancer research. 2006 Oct 1;66(19):9527-34.

32. Gilbert PM, Mouw JK, Unger MA, Lakins JN, Gbegnon MK, Clemmer VB, et al. HOXA9 regulates BRCA1 expression to modulate human breast tumor phenotype. The Journal of clinical investigation. 2010 May;120(5):1535-50.

33. Hayashida T, Takahashi F, Chiba N, Brachtel E, Takahashi M, Godin-Heymann N, et al. HOXB9, a gene overexpressed in breast cancer, promotes tumorigenicity and lung metastasis. Proceedings of the National Academy of Sciences of the United States of America. 2010 Jan 19;107(3):1100-5.

34. Chu MC, Selam FB, Taylor HS. HOXA10 regulates p53 expression and matrigel invasion in human breast cancer cells. Cancer biology & therapy. 2004 Jun;3(6):568-72.

35. Ma XJ, Wang Z, Ryan PD, Isakoff SJ, Barmettler A, Fuller A, et al. A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen. Cancer cell. 2004 Jun;5(6):607-16.

36. Tiffen JC, Bailey CG, Marshall AD, Metierre C, Feng Y, Wang Q, et al. The cancer-testis antigen BORIS phenocopies the tumor suppressor CTCF in normal and neoplastic cells. International journal of cancer Journal international du cancer. 2013 Oct 1;133(7):1603-13.

37. Pilato B, Pinto R, De Summa S, Lambo R, Paradiso A, Tommasi S. HOX gene methylation status analysis in patients with hereditary breast cancer. Journal of human genetics. 2013 Jan;58(1):51-3.

38. Sarno JL, Kliman HJ, Taylor HS. HOXA10, Pbx2, and Meis1 protein expression in the human endometrium: formation of multimeric complexes on HOXA10 target genes. The Journal of clinical endocrinology and metabolism. 2005

Jan;90(1):522-8.
39. Yoshida H, Broaddus R, Cheng W, Xie S, Naora H. Deregulation of the HOXA10 homeobox gene in endometrial carcinoma: role in epithelial-mesenchymal transition. Cancer research. 2006 Jan 15;66(2):889-97.
40. Bromleigh VC, Freedman LP. p21 is a transcriptional target of HOXA10 in differentiating myelomonocytic cells. Genes & development. 2000 Oct 15;14(20):2581-6.
41. Narendra V, Rocha PP, An D, Raviram R, Skok JA, Mazzoni EO, et al. Transcription. CTCF establishes discrete functional chromatin domains at the Hox clusters during differentiation. Science. 2015 Feb 27;347(6225):1017-21.
42. Xu M, Zhao GN, Lv X, Liu G, Wang LY, Hao DL, et al. CTCF controls HOXA cluster silencing and mediates PRC2-repressive higher-order chromatin structure in NT2/D1 cells. Molecular and cellular biology. 2014 Oct;34(20):3867-79.
43. Wang H, Maurano MT, Qu H, Varley KE, Gertz J, Pauli F, et al. Widespread plasticity in CTCF occupancy linked to DNA methylation. Genome research. 2012 Sep;22(9):1680-8.
44. Hur H, Lee JY, Yun HJ, Park BW, Kim MH. Analysis of HOX gene expression patterns in human breast cancer. Molecular biotechnology. 2014 Jan;56(1):64-71.
45. Lee BK, Bhinge AA, Battenhouse A, McDaniell RM, Liu Z, Song L, et al. Cell-type specific and combinatorial usage of diverse transcription factors revealed by genome-wide binding studies in multiple human cells. Genome research. 2012 Jan;22(1):9-24.
46. Ong CT, Corces VG. CTCF: an architectural protein bridging genome topology and function. Nature reviews Genetics. 2014 Apr;15(4):234-46.
47. Stower H. Evolution: Positively selecting CTCF binding. Nature reviews Genetics. 2013 Jan;14(1):6.
48. Li G, Ruan X, Auerbach RK, Sandhu KS, Zheng M, Wang P, et al. Extensive promoter-centered chromatin interactions provide a topological basis for transcription regulation. Cell. 2012 Jan 20;148(1-2):84-98.
49. Chernukhin I, Shamsuddin S, Kang SY, Bergstrom R, Kwon YW, Yu W, et al. CTCF interacts with and recruits the largest subunit of RNA polymerase II to CTCF

target sites genome-wide. Molecular and cellular biology. 2007 Mar;27(5):1631-48.
50. Chen Y, Zhang J, Wang H, Zhao J, Xu C, Du Y, et al. miRNA-135a promotes breast cancer cell migration and invasion by targeting HOXA10. BMC cancer. 2012;12:111.
51. Mustafa M, Lee JY, Kim MH. CTCF negatively regulates HOXA10 expression in breast cancer cells. Biochemical and biophysical research communications. 2015 Nov 27;467(4):828-34.
52. Beygo J, Citro V, Sparago A, De Crescenzo A, Cerrato F, Heitmann M, et al. The molecular function and clinical phenotype of partial deletions of the IGF2/H19 imprinting control region depends on the spatial arrangement of the remaining CTCF-binding sites. Human molecular genetics. 2013 Feb 1;22(3):544-57.

ABSTRACT IN KOREAN

유방암 발생 및 진행에 있어서 CTCF의 기능과 HOX 유전자의 조절자로서의 역할 규명

<지도교수 김명희>

연세대학교 대학원 의과학과

무하마드 무스타파

CTCF (또는 CCCTC-binding factor)는 11개의 징크핑거(zinc finger) 도메인을 가지고 있는 다기능 단백질로서 유전자 좌위 특이적으로 전사의 촉진 또는 억제, 인헨서 블로킹 등의 기능을 수행한다. 다양한 암 종에서 CTCF의 체세포 돌연변이가 발견된다는 점과 CTCF가 세포의 성장, 분화, 세포사멸 등에 관여한다는 점은 암의 진행에 있어서 CTCF가 중요한 역할을 할 것임을 시사

한다. HOX 유전자는 배아 발생 중 형태형성에 있어서 중요한 역할을 담당할 뿐 아니라 성인 세포 내에서도 다양한 세포 내 기능을 조절한다. 따라서 HOX 유전자의 비정상적인 발현이 유방암을 포함한 다양한 암 종에서 많이 발견되고 있다. 본 연구에서는 CTCF가 직접적으로 또는 HOX 유전자의 조절을 통해서 유방암세포의 성장에 미치는 영향을 분석하였다.

CTCF의 과발현(gain of function) 또는 기능억제(loss of function) 연구를 통해서 HOX 유전자 발현에의 영향과 유방암 세포성장에의 영향을 살펴보았다. HOX 유전자 중에서 *HOXA4, HOXC8, HOXA10*이 CTCF에 의해 조절되는 양상을 보였다. 특히 HOXA10은 p53 활성에 영향을 주고 암의 발생과정에 대응하는 역할을 함이 알려져서 새로운 암 억제 유전자로 제시된 바 있다. 여러 암 종에서 암의 발생과 HOXA10의 비활성화와의 관련성을 보여주고 있지만 HOXA10의 조절기전에 대해서는 잘 알려져 있지 않다. 본 연구결과에서는 HOXA10의 프로모터로 예측되는 부위가 전사시작부위로부터 앞쪽으로 5.3-6.1 kb 떨어져 있는 지점에 위치함을 밝혔고 프로모터 기능이 CTCF에 의해 억제됨을 보여주었다. 히스톤 변형패턴 분석을 통해 HOXA10 좌위에서의 CTCF의 존재는 유전자 활성 마커인 H3K4me3의 감소와 유전자 억제 마커인 H3K27me3의 증가를 유도함을 확인하였다. 또한 In silico 분석법과 in vitro 돌연변이 분석 검정을 통해 HOXA10의 중요 프로모터 부위에 CTCF core motif가 중복되어 위치함을 확인하였다. 이상의 결과들로 미루어 볼 때 HOXA10

의 중요 프로모터 부위에 CTCF가 결합함으로써 크로마틴의 환경을 비활성화 시키고 전사인자들의 접근을 방해함으로써 HOXA10 발현을 억제할 것으로 보인다. 유방암세포에서 CTCF에 의한 HOXA10의 후생적 침묵(epigenetic silencing)은 세포주기가 정지됨을 막고 세포사멸을 감소시키고 전이를 촉진시키는 등 종양형성을 진행시키는 요인으로 작용할 것이다.

CTCF의 발현을 억제시킨 경우 세포성장 및 증식의 감소 효과가 정상 유방상피세포주인 MCF10A에서보다 유방암세포주인 MCF-7에서 더 크게 나타났다. 유방암세포에서의 CTCF 발현저해는 직간접적으로 p53 신호 캐스캐이드를 조절할 것으로 보인다. p53 프로모터 부위에의 CTCF 결합은 H3K27me3의 증가와 H3K4me3의 감소를 동반함을 확인하였다. CTCF의 발현을 저해하면 p53의 활성화와 함께 *p21*의 발현이 증가하여 G1/S check point에서의 세포주기 정지, *Bax* 유전자의 발현에 의한 세포사멸의 증가가 유도된다. MCF-7에서 p53이 핵 내에 존재하는 위치는 CTCF의 발현억제와 연관이 있는데 본 연구 결과에서 CTCF 발현억제하에서 p53 신호 캐스캐이드가 활성화됨을 보여주었으며 CTCF에 의한 HOXA10의 전사 억제 효과 역시 p53을 활성화시키는 기전으로 작용함을 확인하였다. 유방암세포주에서 HOXA10은 p53과 발현하는 위치가 같게 나타나는데 HOXA10의 발현저해는 핵내 p53의 감소를 초래하였다. 오토파지 신호경로의 주요 인자인 ATG13, SQSTM1 (P62), LC3-II와 리소좀의 갯수도 CTCF 발현양과 양의 상관관계가 있음을 확인하였다.

이상의 결과들은 CTCF가 직접적으로, 또는 HOXA10을 통해서 p53의 전사억제를 유도하며 이는 유방암세포의 성장 및 발달에 관여함을 보여준다.

핵심되는 말: 유방암, CTCF, HOXA10, p53

PUBLICATION LIST

1. Mustafa M, Lee JY, Kim MH. CTCF negatively regulates HOXA10 expression in breast cancer cells. Biochemical and biophysical research communications. 2015 Nov 27;467(4):828-34.